Habilitation Thesis

Chilling stress in maize
From physiology to genetics and molecular mechanisms

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Chilling stress in maize: From physiology to genetics and molecular mechanisms

A Habilitation Thesis in Crop Science

Presented by
Jörg Leipner

Submitted to the Department of Agricultural and Food Sciences
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This work summarizes our continuous efforts done over the last years to elucidate the physiological and molecular biological mechanisms involved in the chilling and cold stress response of maize seedlings and the genetic sources of tolerance.

The work described here was conducted at the Institute of Plant Sciences of the ETH Zurich in the group of Agronomy and Plant Breeding. I am deeply obliged to Prof. Peter Stamp, head of this group, for his support during the course of this work, but also for always giving me freedom to develop my own ideas. He and Yvan Fracheboud, my doctoral supervisor and later colleague, greatly influenced my scientific thinking and the approaches taken in the research presented here.

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Last but not least, I would like to render homage to the maize god Yumil Kaxob, who was always benevolent with our maize experiments.
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Maize (Zea mays L.) is a crop from the (sub) tropics. During the latter part of the last century, its cultivation has moved within Europe to higher geographical latitudes up to southern Sweden. The expanding maize cultivation in regions with temperate climates still requires breeding for chilling-tolerant maize genotypes and, as a prerequisite, a better understanding of the physiological, genetic and molecular responses of maize to low temperature. The sensitivity of maize to low temperature seems to result primarily from a reduced photosynthetic performance, but probably also due to a decline in metabolite transport. A general overview about the various effects of chilling stress is given in Chapter 1. This overview underlines the necessity of distinguishing between the short-term effects of severe and mild chilling stress and the development of the seedling under mild chilling stress. A short-term exposure of maize seedlings to low temperature results in a reduction of the photosynthetic activity followed by the engagement of dissipative mechanisms and antioxidative systems. These effects, as well as the effects of low temperature on the assimilate transport, are largely reversible. In contrast, development of seedlings at a suboptimal temperature causes a long-lasting perturbation of the photosynthetic machinery. The literature on the genetics of chilling tolerance places a strong focus on the detection of quantitative trait loci (QTLs) for chilling tolerance of photosynthesis; several QTLs have been detected, which are specific for the development of a functional photosynthetic apparatus at low temperature. However according to molecular biological studies, only several genes, which are involved in the response of maize seedlings to chilling stress, have been identified up to now.

In our earlier studies, which are summarized in Chapter 2, it was found that maize grown at suboptimal temperature is characterized by a reduced photosynthetic efficiency and capacity, which seems to be responsible for the reduced growth potential. However, seedlings that developed under this condition could better withstand photooxidative stress and recover faster from photoinhibition than leaves that developed under optimal temperature conditions. In particular, the adjustment of the xanthophyll cycle as well as the increased antioxidative capacity may be the reasons for the better stress tolerance. The comparison of genotypes, however, revealed that chilling-sensitive genotypes are characterized by a higher amount of zeaxanthin and sometimes also by improved scavenging systems in comparison to genotypes with a better chilling tolerance. Seemingly, the higher requirement of dissipative and antioxidative mechanisms in the chilling-sensitive genotypes grown under suboptimal temperature is responsible for this effect. Consequently, it seems that differences in the dissipative and/or antioxidative mechanisms cannot explain the genotypic level of chilling tolerance without an exact definition of the circumstances, i.e. stress severity including the previous acclimation history.

In order to find the primary causes of chilling tolerance/sensitivity in maize seedlings, first their genetic causes have to be elucidated; thereupon, the molecular and physiological mechanisms of chilling tolerance can be unraveled based on the results obtained by the genetic studies. The quantitative trait locus (QTL) analysis was chosen to study the genetic background of chilling tolerance. This method requires an appropriate plant material. Optimal research material would be a segregating population with parents that are characterized by contrasting chilling tolerance. Such inbred lines with contrasting chilling tolerance had to be developed. Firstly, a method based on chlorophyll fluorescence analysis was developed to efficiently discriminate maize genotypes of contrasting chilling tolerance of photosynthesis (Chapter 3). By using inbred lines of known chilling-tolerance, photosynthetic parameters were determined at different temperatures in seedlings that had developed at optimal and suboptimal growth temperatures. When seedlings were raised at suboptimal temperature (15 °C), the PSII operating efficiency ($F_{v'}/F_{m'}$) measured at low temperature (6 °C) and low light intensity (50 µmol m$^{-2}$ s$^{-1}$) allowed to efficiently discriminate the chilling-tolerant genotypes from the chilling-sensitive ones. Applying this methodology enabled divergent selections by $F_{v'}/F_{m'}$ within three populations, two major Swiss ones (dent and flint) as well as a partially exotic one, developed previously by us from Latin American highland germplasm. This approach was successful and led to an increase or decrease in the carbon exchange rate of hybrids derived from divergently selected inbred lines. The obtained inbred lines allowed us to generate mapping populations, which segregate for chilling tolerance of photosynthesis.
The focus was made on dent material as this is generally less chilling-tolerant than flint material; together both germplasm pools are still generating the major part of the Central European commercial hybrids. By crossing two dent maize lines with contrasting chilling tolerance of photosynthesis, a mapping population was generated. It was then analyzed under controlled conditions for quantitative trait loci (QTLs) associated with the chilling tolerance of photosynthesis (Chapter 4). Several genomic regions were revealed, which are important for the chilling tolerance of photosynthesis. Only a few QTLs were clearly identified in both temperature regimes for the same traits, indicating a constitutive expression. This demonstrated that the genetic control of the performance of the photosynthetic apparatus was mostly temperature specific. A major QTL for the chilling tolerance of photosynthesis was identified on chromosome 6. This QTL alone explained a large part of the phenotypic variation in the maximum quantum efficiency of PSII primary photochemistry ($F_v/F_m$). It was also significantly involved in the expression of six other traits, including the rate of carbon fixation and shoot dry matter accumulation. The latter indicates that the tolerance of the photosynthetic apparatus to chilling is a key factor for the growth of maize seedlings at low temperature. A further major QTL for photosynthesis-related traits, which was detected on chromosome 2, corresponded to a QTL identified previously in a tropical maize population which was developed by CIMMYT (Mexico).

As these QTLs stemmed from seedlings in strictly controlled environments, their relevance was studied under field conditions in the same mapping population (Chapter 5). By sowing the population both at an early and a medium late date each year, seedlings were exposed both to unfavorable and more favorable temperature conditions. The main QTLs involved in the functioning of the photosynthetic apparatus at low temperature were stable across the unfavorable chilly environments and were mostly identical with the QTLs detected under controlled conditions at suboptimal temperature. In contrast to the growth at controlled conditions, the functioning of the photosynthetic apparatus in plants developed at low temperature in the field did not noticeably affect biomass accumulation. Furthermore, there were no co-localizations between QTLs for leaf area and shoot dry weight. Consequently, biomass accumulation did not seem to be carbon-limited at the autotrophic seedling stage under chilly conditions in the field.

Confirmation of the relevance of early chilling tolerance on later field performance of maize is largely lacking. By letting part of the seedlings grow on until flowering and maturity, such relationships could be studied with our dent mapping population, including QTL analyses at early development, at flowering and at silage maize maturity (Chapter 6). Across environments (years and sowing dates), significant stable QTLs were found for plant height and for the time of flowering. The QTLs for flowering were frequently co-localized with QTLs for plant height or ear dry weight. However, the focal comparison with QTLs detected at seedling stage revealed only few common QTLs. A pleiotropic effect was found on chromosome 3; a good photosynthetic performance of the seedling under warm conditions had a beneficial effect on plant height and partially on biomass at harvest. However, a high chilling tolerance of the seedling seemingly had an insignificant or small negative effect on the yield. This really demands follow-up studies in diverse mapping populations tested in diverse environments in order to conclude on the importance of early seedling vigor for future growth stages.

The QTL analysis is a powerful tool not only to identify genomic regions responsible for the expression of a trait, but also for unraveling interactions among traits. Based on this, an in-depth analysis of the relationships between chlorophyll fluorescence parameters was conducted in a further QTL experiment (Chapter 7). Additionally, by growing the mapping population also under high light intensity, this study allowed gaining information about the role of excitation pressure in the expression of the major QTLs for chilling tolerance of photosynthesis. By dissecting the chlorophyll fluorescence parameters, it was shown that the PSII operating efficiency ($F_m'/F_n'$) could be independently modulated by the PSII maximum efficiency ($F_v'/F_m'$) and the PSII efficiency factor ($F_v/F_m$). In particular, under high excitation pressure, the PSII operating efficiency ($F_m'/F_n'$) was mainly controlled by its maximal efficiency ($F_v'/F_m'$) whilst components down-stream of $P_{680}$ additionally modulated the PSII operating efficiency under low excitation pressure. Moreover, genotypic differences in the maximum quantum efficiency of PSII photochemistry ($F_v/F_m$) caused by growth at suboptimal temperature could be attributed to both persistent down-regulation of PSII and to damage of the PSII reaction center.

The role of the temperature at night in the expression of QTLs for photosynthesis-related traits was studied in a publicly available dent mapping population, which was derived from the cross between Mo17 and B73, the so called IBM302 population (Chapter 8). It was grown under optimal temperature (24/22 °C) or under suboptimal temperature (17 °C) with temperatures at night of 13 °C and 6 °C, respectively. The study disclosed that the temperature at night affects photosynthesis-related traits in a genotype dependent manner. The major QTL for photosynthesis-related traits, which was localized on chromosome 5, showed a lower phenotypic variance and a smaller additive effect when seedlings were exposed to the lower temperature of 6 °C rather than 13 °C at night. Since only few minor QTLs were detected in only one of the two tested temperatures at night, the QTL at chromosome 5 seems largely to be responsible for the contrasting response
of the parental lines to the temperature at night. The genes *ivr2*, coding for a vacuolar acid invertase, and *a2*, coding for an anthocyanidin synthase, were found to map near this major QTL. Based on their potential involvement in the acclimation to low temperature, these two genes were identified as potential candidate genes. However, verification of these candidates by performing QTL analyses for the activity of soluble acid invertase and for the content of anthocyanin did not reveal QTLs for these traits at chromosome 5, indicating that neither *ivr2* nor *a2* is the underlying gene of this QTL. Nevertheless, it was found that a major QTL for invertase activity is located nearby the gene *ivr1* and the major QTL for anthocyanin content maps close to the locus *ri1*, which is known to be involved in the regulation of anthocyanin content. Consequently, the IBM302 population seems to be of high value for studying the genetics of these traits in more detail. Comparative mapping here revealed that certain QTLs were present across maize mapping populations, when similar traits were considered and plants had grown under comparable conditions. The C4 cycle enzymes and the foliar antioxidants have long been discussed as being key factors in the chilling tolerance of maize seedlings. In order to elucidate such roles, their activities and amounts were analyzed in the dent mapping population grown under suboptimal temperature and compared with QTLs found for chilling-tolerance of photosynthesis (Chapter 9). Significant QTLs were detected for the extractable activity of phosphoenolpyruvate carboxylase, malate dehydrogenase and malic enzyme as well as for the amounts of ascorbate and α-tocopherol. These QTLs, however, were not co-localized with the major QTLs for photosynthesis-related traits, indicating that the C4 cycle enzymes and the studied antioxidants play a minor role in the genotypic differences in chilling tolerance of photosynthesis. However based on this QTL analysis, a strong relationship was found between the chlorophyll *a*/*b* ratio and the chilling tolerance of photosynthesis. This indicates that the assembly of the photosynthetic apparatus seems to be of higher relevance for chilling tolerance than processes down-stream of photosynthesis.

The major QTLs for chilling tolerance of photosynthesis were also found to be involved in the specific leaf area (SLA), i.e. the leaf area produced by a unit of dry matter (Chapter 10). By performing QTL analyses of this as well as of an alternative dent mapping population, which was provided by the Experimental Institute for Cereal Crops (Bergamo, Italy), and by examining several *Sv* inbred lines, which were previously generated by divergent selection for high or low *Fv'/Fm'* at suboptimal temperature. This close negative relationship was not present in seedlings grown at optimal temperature. Whereas a high SLA is a desirable trait for breeding plants with good growth performance, as it guarantees an early ground cover at the same dry matter investment by a larger leaf area, it seems to be the consequence of an incapability of chilling-sensitive genotypes grown at suboptimal temperature to increase their SLA probably due to a reduced availability of assimilates. Furthermore, these results demonstrated that selecting for high *Fv'/Fm'* at suboptimal temperature did not lead to a constitutively altered SLA. In these QTL studies genetic regions could be identified, which are involved in the chilling tolerance of photosynthesis. For these genetic regions, positional candidate genes were identified and then narrowed down by functional analysis mainly to genes directly or indirectly involved in the photosynthetic light reaction. However, the QTL analyses did not allow to verify that these genes are indeed responsible for the expression of the QTLs for chilling tolerance of photosynthesis. Consequently, molecular analyses were conducted to get further and more detailed insight in the response of maize seedlings to low temperature. For this purpose cold-induced genes were analyzed using the PCR-select cDNA subtraction method (Chapter 11). This method allowed to isolate several novel genes from maize seedlings exposed for 48 hours to 6 °C. Of 18 *Zea mays* cold-induced genes (*ZmCOI*) genes characterized, the majority shared similarities with proteins of known function in signal transduction and photosynthesis regulation. For a selected group of genes, RT-PCR analysis confirmed the induction by low temperature. This selected group consisted of genes that are involved in Ca2+ signaling (*ZmAIA1*) and in the regulation of transcription (*ZmDREB2A* and *ZmERF3*) as well as the new gene *ZmCOI* that was chosen due to its high frequency in the subtracted cDNA library. In addition, it was found that the expression of these genes was strongly induced by other abiotic stresses such as drought and high salt concentration, by stress signaling molecules such as jasmonic acid, salicylic acid and abscisic acid and also by membrane rigidification. These results suggested that this group of genes is involved in a general response to abiotic stresses. Among these genes, the novel gene *ZmCOI* was studied in more detail (Chapter 12). The predicted *ZmCOI* amino acid sequence and its homologue were found to be very similar to proteins in rice and *Arabidopsis*, suggesting that it belongs to a conserved group of plant proteins. Analysis of the *ZmCOI* promoter sequence revealed several conserved stress-responsive cis-acting elements, which is in accordance with the finding that *ZmCOI* is induced by other abiotic stresses and signaling molecules. Another characteristic of *ZmCOI* is its alternative splicing; it yielded two transcripts, the level of which changed depending on the stress, indicating a possible mechanism of regulation at the splicing level. Constitutive expression of *ZmCOI* in *Arabidopsis* yielded plants with a
lower level of abiotic stress tolerance, giving evidence that ZmCOI6.1 may be a negative regulator.

The conclusions based on these studies are presented in Chapter 13. The main focus is on the development of a functional photosynthetic apparatus under low temperature. Hereby, the most crucial point seems to be the incapability of maize seedlings to generate a functional PSII reaction center while the assembly of the light harvesting complex appears to be little affected by low temperature. Based on the QTL analyses, no evidence has been found that antioxidants, C₄ cycle enzymes or anthocyanin play an important role for genotypic differences in chilling tolerance of photosynthesis. However, the large genetic variation (including maternal effects) for chilling tolerance across the maize germplasm as well as its quantitative nature makes it difficult to pinpoint a particular molecular mechanism as the primary cause of chilling sensitivity in maize. The results of the QTL experiments allowed concluding that effects of chilling tolerance of photosynthesis on growth are strongly dependent on environmental conditions. At low or medium light intensity, chilling tolerance of photosynthesis has an effect on biomass accumulation. However under field conditions, it hardly affects seedling growth and has seemingly no influence on yield. In contrast, the photosynthetic performance under optimal temperature appears to be more important for yield. Nevertheless, chilling tolerance of seedlings will also play an important role in the future to permit early planting in order to avoid potential drought stress during flowering and to guarantee a healthy early development. Although interactions on the molecular and the physiological level exist, a separate treatment of chilling tolerance and of drought tolerance has to be respected since they are due to different genetic backgrounds.
Maize is very sensitive to chilling especially during early autotrophic growth. Seemingly, photosynthesis is strongly affected due to the inhibition of certain enzymes of the C₄ and the Calvin cycle. Cold-induced perturbations of phloem loading may have negative feedback effects on photosynthesis, too. The reduced photosynthetic activity promotes dissipative mechanisms and affects the antioxidative defense in maize leaves. Although seedlings can withstand chilling stress without visual symptoms for few days, the development under such conditions results in irreparable damages with developing chloroplasts and the leaf meristem as the first targets. However, development at suboptimal temperature enables seedlings to better withstand further stress probably due to improved dissipative and antioxidative mechanisms. The causal physiological mechanisms for a better chilling tolerance remain still largely unknown; but recently, first QTLs for chilling tolerance of maize seedlings have been identified. Together with the growing amount of information from gene expression studies this may help to finally unravel the mechanism of chilling tolerance.

As a tropical plant, maize is sensitive to low temperature, despite its origin at altitudes of about 2000 m.s.l. Low temperature prolongs growth duration, reduces crop growth rate, and thus weakens the seedling; at the end of the growth cycle frost may terminate grain filling prematurely. Consequently, grain yields are inconsistent and often lower in temperate climates or mountainous regions. Ensuring optimal conditions for germination have been recommended to alleviate the impact of cold on early crop development. At the same time, however, requirements to achieve sustainable soil protection by mulch or no-till systems, which decrease soil temperature and depend on increasingly chilling-tolerant genotypes, are being enforced. Maize germinates at temperatures below 10 °C. Therefore, a major improvement in low-temperature germination makes sense only if the seedlings are also chilling-tolerant. Maize seedlings are most sensitive during the transition phase from heterotrophic to autotrophic growth. This indicates that C assimilation is often more affected by low temperature than metabolic processes. High chilling tolerance during autotrophic growth is accompanied by a high relative growth rate, sustained by a high net assimilation rate in spite of a low leaf area ratio. Thus, the emphasis must be placed on photosynthesis. To avoid misinterpretation of the results, it is necessary to define the types of chilling stress. Here cold refers to temperature below 5 °C, when neither growth nor photosynthesis occurs and the plant depends on defense mechanisms to avoid damage and to survive. Chilling occurs at 5 to about 15 °C, when plants are still capable of adapting developmental processes in order to survive more unfavorable temperature conditions like during cold spells. As in the literature, optimal temperature is the temperature between 20 and 30 °C at which plants develop rapidly; at suboptimal temperature (about 15 °C) plants can acclimate rapidly but growth is retarded. Furthermore, the developmental stage of the plant must be defined. For example, during the early seedling stage, the leaf development depends strongly on soil temperature as the apex is located a few centimeters below the soil surface. Although cold spells lasting a few minutes to several hours have received a great deal of attention, it is now clear that chilling periods lasting a few days to weeks are more important during seedling establishment since farmers sow at soil temperatures above 8 °C; at this time of the season, mean temperatures are at the acclimating level of about 15 °C.

Physiological effects of short-term low-temperature stress

Plants sense temperature through changes in membrane rigidity, which cause an increase in the cytoplasmic concentration of Ca²⁺, which functions as a second messenger in plant signaling. Investigations of the cold-induced Ca²⁺ signal in maize have revealed that, in comparison to cold-tolerant wheat, maize cannot quickly restore the cytoplasmic Ca²⁺ concentration, probably due to a decrease in Ca²⁺-ATPase activity (Jian et al., 1999). A permanently high cytoplasmic Ca²⁺ concentration might lead to
damage to the cell. Another sensor of low temperature is the redox state of the cell, which is strongly affected by photosynthetic activity. Disturbance of photosynthesis through low temperature causes the greatest harm because a chilling-induced slow-down of enzymes or reactions involved in photosynthesis affects the redox state and increases the risk of damage to the cell by reactive oxygen species.

**Effects of chilling on photosynthesis and down-stream processes**

The most obvious response of maize seedlings exposed to low temperature is the reduction of photosynthesis. Several reactions, from the photosynthetic light reaction to the sink of assimilates, are considered to be the main effects of chilling stress. Temperature per se seems to have little effect on the photosynthetic light reaction, as was demonstrated by infiltrating maize leaves with an artificial electron acceptor (Fracheboud and Leipner, 2003). As a result, the fluidity of the thylakoid membrane, known to be affected by temperature (Barber et al., 1984), does not seem to disturb the photosynthetic electron transport chain.

Low temperature affects plants primarily by decreasing the velocity of enzymatic reactions and, consequently, the photosynthetic dark reaction is potentially affected by cold. In maize, the reduction of photosynthetic capacity upon exposure to low temperature might be caused by a decrease in the activity of certain enzymes of the C4 cycle and/or of the Calvin cycle. For example, Kingston-Smith et al. (1997) observe lower activity of NADP malate dehydrogenase and Rubisco in maize exposed to cold. In the C4 plant *Sorghum bicolor* the maximum steady state rate of CO2 fixation by the enzyme phosphoenol pyruvate carboxylase was limited by the re-synthesis of phosphoenol pyruvate (Laisk and Edwards, 1997). This re-synthesis, i.e. the phosphorylation of pyruvate to phosphoenol pyruvate, is catalyzed by the enzyme pyruvate orthophosphate dikinase (PPDK). Even when precaution was taken to maximize the recovered activity, it has been difficult to demonstrate a higher PPDK activity in leaf extracts from C4 plants than the maximum rate of photosynthesis of those leaves (Usuda et al., 1984a). This, combined with the fact that the enzyme is subjected to a complex mechanism of dark/light-based regulation, has led to the assumption that the PPDK reaction is a key site of regulation of C4 photosynthesis. Other results indicate that the photosynthetic flux in C4 plants is predominantly controlled by Rubisco and, to a lesser extent, by PPDK (Sage and Kubien, 2007). Rubisco does not seem to be particularly sensitive to low temperature, but the light-induced increase in its maximum catalytic activity was slowed down under cold conditions, suggesting that circadian- and light-regulated transcription and synthesis of Rubisco were impaired (Kingston-Smith et al., 1997). Transformation of maize with PPDK from *Flaveria brownii*, a C4 plant, which is known to be more cold-tolerant than maize, resulted in a greater cold tolerance of extractable PPDK than in the wild type (Ohta et al., 2004). However, measurements of photosynthesis in vivo showed only marginal differences between transformants and the wild type (Ohta et al., 2006). This may be due to the fact that control of the photosynthetic flux seems to be shared by Rubisco and PPDK.

Although exposure to low temperature results in a decline in the assimilation rate, there was a strong increase in the sugar and starch contents after several days at chilling temperature (Sowinski et al., 1999), indicating effects of chilling downstream of the Calvin cycle. By means of radio labeling, Sowinski et al. (1999) indicated a slowdown in the transport of photoassimilates from the site of synthesis to the phloem. Furthermore, phloem loading is strongly affected by chilling temperature, especially in a chilling-sensitive dent as compared to a chilling-tolerant flint genotype (Sowinski et al., 1998). Difference in phloem loading capability at chilling temperature between ssp. *indentata* and ssp. *indurata* may be due to structural differences in the vascular bundles (Sowinski et al., 2001). Although phloem loading in maize is apoplastic, the high number of plasmodesmata between donor cells and companion cells in seedlings of the dent type indicate that the companion cell/thin-walled sieve tube complex is not isolated symplesmatically from the adjoining cells. Since symplasmic transport is particularly sensitive to low temperature, the lower chilling tolerance of ssp. *indentata* was explained by structural differences (Sowinski et al., 2001). As well as the cold-induced slowdown of phloem loading, the transport speed of assimilates in the phloem also decreases when temperature is lowered. However, since the decrease in phloem transport was the same order of magnitude as the decrease in photosynthesis, the accumulation of photosynthates in the leaves does not seem to be due to a decrease in phloem transport (Sowinski et al., 1999).

It is obvious that chilling affects the photosynthesis of maize seedlings down-stream of the photosynthetic light reaction. Wherever the location of this primary site is, a coordination between the photosynthetic electron transport and the activity of carbon metabolism is a prerequisite of minimizing the risk of the generation of reactive oxygen species (ROS). The chilling induced slowdown of the Calvin cycle, be it directly or indirectly by negative feedback mechanisms, results in a depletion of ADP and, consequently, in the formation of a large ΔpH across the thylakoid membrane. The lower pH in the thylakoid lumen triggers the down-regulation of photosystem II by the formation of non-photochemical...
quenching (NPQ) and by the de-epoxidation of violaxanthin via antheraxanthin to zeaxanthin (Szabó et al., 2005). In maize seedlings, exposure to cold in the light results in the rapid formation of NPQ followed by de-epoxidation of the xanthophyll cycle pool (Leipner et al., 1997). The importance of the xanthophyll cycle for photoprotection has been demonstrated in mutants lacking this pigment (Pasini et al., 2005). However, faster de-epoxidation of the xanthophyll cycle pool did not result in a greater cold tolerance of maize seedlings (Leipner et al., 2000b).

The role of antioxidants
The loss of metabolic homeostasis due to adverse environmental factors results in a greater production of reactive oxygen species (ROS) (Suzuki and Mittler, 2006). Exposure of maize seedlings to cold in the dark resulted in a transient increase in hydrogen peroxide (H$_2$O$_2$) (Prasad et al., 1994). Furthermore, the chilling induced reduction in photosynthetic activity increases the risk of ROS generation in the event that excess absorbed light energy cannot be dissipated as heat. Therefore, accumulation of ROS is thought to occur when maize seedlings are exposed to low temperature. Furthermore, it was observed that the accumulation of superoxide radicals (O$_2^-$) in maize leaves after exposure to light becomes more intense as temperature decreases (Ke et al., 2004). However, due to the high reactivity of ROS and consequent short lifespan, the generation of ROS is difficult to quantify. Thus, our knowledge of this fundamental process is incomplete with respect to maize under chilling stress.

Plants possess non-enzymatic and enzymatic scavenging systems, which should keep ROS at a level that is not harmful. Scavenging enzymes operate as ROS scavengers or, rather, they are involved in recycling antioxidants. At the heart of the enzymatic scavenging system stays the so called water-water cycle (Asada, 1999). In the water-water cycle, O$_2^-$, which can be generated by the reduction of molecular oxygen at photosystem I by means of the Mehler reaction, is detoxified by a pathway of enzymatic reactions. In the first step, superoxide dismutase catalyzes the dismutation of O$_2^-$ to yield H$_2$O$_2$. In the chloroplast, H$_2$O$_2$ is detoxified by ascorbate peroxidase with ascorbate as the hydrogen donor. This catalytic removal of H$_2$O$_2$ results in the formation of monodehydroascorbate, which can be reduced enzymatically to ascorbate by monodehydroascorbate reductase, with both NADH and NADPH as electron donors. Alternatively, monodehydroascorbate can decay non-enzymatically by spontaneous disproportionation to ascorbate and dehydroascorbate. The enzyme dehydroascorbate reductase catalyzes the reduction of dehydroascorbate by the reduced form of glutathione (GSH) in the chloroplast stroma. The re-reduction of oxidized glutathione (GSSG) is catalyzed by glutathione reductase with NADPH supplying electrons.

A decrease in temperature affects the velocity of most of these reactions. In particular, the activity of glutathione reductase and especially that of dehydroascorbate reductase was lower under cold temperature (Jahnke et al., 1991). For glutathione reductase, a decrease in the assay temperature resulted in a strong decrease in $V_{\text{max}}$, while $K_m$ was less affected (Hull et al., 1997). For ascorbate peroxidase, cold also resulted in a decrease in $V_{\text{max}}$ but $K_m$ increased considerably (Hull et al., 1997). At optimal temperature, the activity of glutathione reductase and ascorbate reductase are higher in chilling-tolerant than in chilling-sensitive genotypes or species of the genus Zea (Hull et al., 1997; Jahnke et al., 1991; Kocsy et al., 1996).

Ascorbate and glutathione are the two major antioxidants in plant cells. Whether or to which extent chilling stress affects maize seedlings by depleting or oxidizing these antioxidants seems to depend largely on the studied stress conditions and the developmental stage of the plant (Hodges et al., 1996; Leipner et al., 1997; Leipner et al., 2000a). A clear correlation between the level of antioxidants in plants grown at optimal temperature or after a short-term chilling stress with the chilling tolerance of the genotype has not been found (Hodges et al., 1996). Although the amount of ascorbate is usually about ten times higher than that of glutathione in leaves of maize seedlings (Leipner et al., 1997) glutathione seems to play a central role in the redox system of the cell; the GSH/GSSG ratio may function as an ubiquitous regulatory signal (Foyer and Noctor, 2005). The importance of glutathione is supported by the observation that inhibition of glutathione synthesis reduced the chilling tolerance of maize seedlings (Kocsy et al., 2000) while an increase in glutathione synthesis by means of herbicide safeners resulted in a greater chilling tolerance (Kocsy et al., 2001). An artificial increase in ascorbate did not affect tolerance to cold-induced photoinhibition, although, at the same time, it accelerated the de-epoxidation of the xanthophyll cycle pool (Leipner et al., 2000b).

Due to the C$_4$ syndrome in maize, antioxidants are differentially localized between mesophyll and bundle sheath cells (Doulis et al., 1997). Bundle sheath cells are characterized by high amounts of superoxide dismutase as well as of ascorbate peroxidase. These enzymes are absent in mesophyll cells. On the other hand, mesophyll cells contain glutathione reductase and dehydroascorbate reductase, which are absent in bundle sheath cells. Consequently, the amount of reduced ascorbate and glutathione is higher in mesophyll than in bundle sheath cells. This lack of antioxidants might explain the greater susceptibility of the bundle sheath cells to oxidative and chilling stress (Kingston-Smith and Foyer, 2000a). It is
noteworthy that glutathione reductase transcripts are found in both types of cells but are translated to its protein only in mesophyll cells (Pastori et al., 2000b). However, *de novo* synthesis of glutathione can occur in both cell types (Foyer et al., 2002). After exposure to chilling, the content of GSSG increased in the mesophyll as well as in the bundle sheath cells, but the GSH/GSSG ratio decreased in the bundle sheath cells only (Kopriva et al., 2001).

### Physiological and developmental effects of the growth of maize seedlings at suboptimal temperature

The effects of a sudden temperature decrease on physiological processes of maize seedlings, especially on the photosynthesis and antioxidative systems, were investigated extensively under controlled growth conditions. In fact, under field conditions in temperate regions of Europe, maize is exposed permanently to suboptimal rather than to optimal temperature with the occurrence of sudden cold spells. With respect to the physiology and the development of the maize seedling, the effects of suboptimal growth temperature are quite different from the effects of a sudden short-term decrease to low temperature. Suboptimal growth temperature is considered to be a moderate stress under which growth occurs but nonetheless disturbs metabolism and development.

When maize is grown in the field, the photosystem II operating efficiency *(F₀/F₉)* decreases during periods of low temperature (Andrews et al., 1995). Because phases of low temperature lead to a sustained reduction in photosynthesis (Stirling et al., 1993) and affect photosynthesis especially in young leaves (Stirling et al., 1991), chilling seems to have a strong effect on the development of the photosynthetic machinery.

#### Primary sites affected by suboptimal growth temperature

The observation that fully developed leaves are less sensitive to chilling than developing leaves indicates differences in the response of the tissues to chilling, depending on the developmental stage. The shoot apex is very sensitive to low temperature, as shown by seedlings whose root zone was cooled down. As well as a negative effect on the growth rate and leaf expansion (Engels, 1994; Stone et al., 1999) a decrease in the temperature of the root zone also had a negative effect on the photosynthetic performance of maize seedlings, especially in chill-sensitive genotypes (Chassot, 2000; Hund, 2003). In other experiments, however, cooling of the shoot apex resulted in a delay in leaf development but did not sig-

![Figure 1: Cold-induced damage to the third leaf of maize seedlings developed during and after four days at 6 °C and 400 µmol m⁻² s⁻¹. *Left:* Spatial location of chlorosis (black bars). The position of the chlorotic area, which became visible after recovery at 25 °C, was used to localize the tissue that was most susceptible to chilling (gray bars). *Right:* Damage to the photosystem II reaction center determined by chlorophyll fluorescence images of the minimum fluorescence *(F₀)* and of the maximum quantum efficiency of photosystem II primary photochemistry *(F₉/F₉)* after recovery at 25 °C. High values (red) of *F₀* and low values (blue) of *F₉/F₉* indicate damage to photosystem II. The cold-induced damage on the third leaf originated from the leaf tissue which was positioned, at the time of the cold treatment, at the height of the base of the second leaf (upper arrow) and at 1 to 2 cm above soil level (soil level indicated by lower arrow) (J. Leipner, unpublished results).
nificantly affect the efficiency of photosynthesis (Sowinski et al., 2005). The cold-induced growth retardation of the leaves seems to be caused by a strong increase in the duration of the cell cycle and by a reduction of cell production when seedlings are exposed to low temperature (Rymen et al., 2007). Spatial analysis of leaf blades, which were exposed to cold (6 °C, 400 µmol m⁻² s⁻¹) for four days, revealed two zones with chlorosis (Figure 1). These areas could be tracked back to the meristem of the leaf and the region of the leaf that had just been exposed to light. It is concluded that (i) chilling induces damage during cell production, which prevents the development of fully functional cells, and (ii) the combination of light and low temperature leads to the disturbance of chloroplast development.

**Development of the photosynthetic apparatus under chilling conditions**

Suboptimal growth temperature results in a decrease both in the capacity and efficiency of photosynthesis (Nie et al., 1992), especially in chill-sensitive genotypes (Fracheboud et al., 1999). The weaker photosynthetic performance persists if seedlings that developed under suboptimal temperature are examined under optimal temperature (Haldimann, 1996; Nie et al., 1992), indicating the occurrence of structural changes during the development of the photosynthetic machinery. Moreover, the photosynthetic activity of leaves that developed under suboptimal temperature and were afterwards exposed to optimal temperature for several days is lower than that of leaves developed continually at optimal temperature (Nie et al., 1995). In contrast to photosynthetic activity, which recovers only slightly after transfer to optimal temperature, the chlorophyll content of these leaves reached almost the chlorophyll content of leaves, which developed continually at optimal temperature. First conclusions about the nature in the changes of the photosynthetic machinery due to suboptimal growth temperature can be drawn from analyses of the pigment composition. Pigment analyses revealed a strong decrease in the chlorophyll a/b ratio at suboptimal growth temperature, especially in chill-sensitive genotypes (Haldimann et al., 1995; Haldimann, 1998). Since most of chlorophyll b is found in the antenna proteins, especially in the light harvesting complex II (LHClII), these results indicate that the ratio of LHClII to the reaction centre (RC) increases at suboptimal growth temperature. A detailed analysis by means of sucrose gradient fractionation and SDS-PAGE confirmed this hypothesis (Caffarri et al., 2005). Furthermore, the number of minor antenna complexes decreased in comparison to LHClII when maize seedlings grew at suboptimal compared to optimal temperature but to a lesser extent than RCII. In particular, plastid-encoded proteins of the reaction centers fail to accumulate in the mesophyll thylakoids when seedlings develop at suboptimal temperature (Nie and Baker, 1991). This lack of certain thylakoid proteins was found in the chloroplasts of the mesophyll and the bundle sheath (Robertson et al., 1993). The chill-induced reduction of the accumulation of certain thylakoid proteins is also reflected by the atypical ultrastructure of the mesophyll chloroplasts. Mesophyll chloroplasts from leaves that developed at suboptimal temperature are slightly bigger and more round in shape (Kutik et al., 2004; Robertson et al., 1993). Furthermore, extensive vesiculation occurs at low temperature, especially in maize genotypes sensitive to chilling, which results in the disruption of the granal array (Pinhero et al., 1999). Furthermore, growth at suboptimal temperature affects the fatty acid composition of the chloroplast membranes, with an increase in unsaturated fatty acids (Fracheboud, 1999).

With regard to the activity of enzymes from the C₄ cycle and the Calvin cycle, a lower content of Rubisco and phosphoenolpyruvate carboxylase was found in leaves that developed at suboptimal temperature, while the NADP malate dehydrogenase activity increased compared to leaves grown at optimal temperature (Kingston-Smith et al., 1999). The chill-induced reduction in Rubisco amount was more pronounced in a chill-sensitive than in a chill-tolerant genotype (Pietrini et al., 1999). However, the chill-induced decrease in the maximal Rubisco activity can be compensated by an increase in its activation state (Kingston-Smith et al., 1999). Due to the C₄ syndrome, assimilates must be transported between the mesophyll and the bundle sheath cells. Leaf development at suboptimal temperature leads to a larger area between the cells of the mesophyll and the bundle sheath and to a higher number of plasmodesmata between these cell types (Sowinski et al., 2003). This acclimation seems to compensate for the chill-induced slowdown of diffusion from cell to cell but was less distinct in chill-sensitive compared to a chill-tolerant genotype.

**Consequences of chill-induced changes in the photosynthetic machinery**

Since pigment protein complexes as well as other components in the thylakoid membrane are not properly assembled when maize seedlings develop at suboptimal temperature, a safe operation of photosynthesis is more difficult. Similar to short-term chilling stress, growth at low temperature results, even at low light intensity, in an excess of absorbed light energy. Therefore, it is not surprising that growth at suboptimal temperature results in the accumulation of xanthophyll cycle pigments in the leaves (Haldimann et al., 1995). Furthermore, the xanthophyll cycle pool can be de-epoxidized to a greater extent in leaves grown at suboptimal than at optimal...
temperature (Leipner et al., 1997). The lower photosynthetic activity of chill-sensitive genotypes relies on a more efficient dissipation of the excess absorbed light energy as heat; therefore, these genotypes are characterized by higher amounts of xanthophyll cycle pigments (Haldimann, 1998). It is assumed that suboptimal temperature increases the generation of ROS and, in consequence, causes damage to biological structures. Increasing amounts of H$_2$O$_2$ were found with decreasing growth temperature (Kingston-Smith et al., 1999). This increase in ROS was particularly pronounced in mesophyll cells, which are characterized by a higher H$_2$O$_2$ content compared to bundle sheath cells (Pastori et al., 2000a). Furthermore, under unfavorable growth conditions in the field, there may be a surplus of ROS (Fryer et al., 1998). However, chill-induced disturbance of the development of the photosynthetic apparatus under low-temperature conditions in the field are not necessarily associated with O$_2$ production through the Mehler reaction (Leipner et al., 1999a). Nevertheless, the increases in the amount of antioxidants and in the activity of scavenging enzymes indicate that seedlings are under an oxidative stress when they grow at suboptimal temperature. The contents of the three major foliar antioxidants, namely ascorbate, glutathione and $\alpha$-tocopherol, increased when plants developed at suboptimal temperature (Kingston-Smith et al., 1999; Kocsy et al., 1996; Leipner et al., 1997). The situation is less clear for enzymes involved in the water-water cycle. Suboptimal growth temperature resulted in a higher activity of superoxide dismutase and glutathione reductase (Hull et al., 1997; Leipner et al., 2000a; Massacci et al., 1995). In another study, however, the activity of all the enzymes of the water-water cycle, with the exception of superoxide dismutase, was lower at a growth temperature of 14°C than at 18°C (Kingston-Smith et al., 1999). It is clear that the activity of catalase is largely lower in leaves that developed at suboptimal temperature (Kingston-Smith et al., 1999; Leipner et al., 2000a). The importance of ROS in the stress response is still not fully understood, especially since ROS, like H$_2$O$_2$, are important signaling molecules (Foyer et al., 1997) and seem to be essential for growth processes (Rodríguez et al., 2004). The question also remains as to whether the weaker photosynthetic performance of chill-sensitive genotypes is reflected in a lower biomass accumulation compared to chill-tolerant genotypes. In a comparison of a chill-tolerant (Z7) and a chill-sensitive genotype (Penjalinan), Verheul et al. (1995) found an association between good photosynthetic performance and a QTL for photosynthetic performance and a QTL for shoot dry matter in seedlings grown at suboptimal temperature (Fracheboud et al., 2004; Hund et al., 2004). However, this pleiotropic effect was not present when plants developed at optimal temperature. Furthermore, such a co-localization was not present under chilling conditions in the field, neither when seedlings developed under chilling conditions in early spring, nor when they grew later in the season at more optimal temperature (Jompuk et al., 2005). It seems that photosynthesis limits seedling growth only at low temperature combined with low to medium light intensity. While the persistent depression of photosynthesis at suboptimal growth temperature can have a negative effect on the biomass accumulation of the maize seedlings, it has the beneficial effect that seedlings grown under such conditions are more tolerant to cold-induced photooxidative stress (Leipner et al., 1997) and recover faster from photoinhibition (Haldimann et al., 1996). Furthermore, in some genotypes, seedlings that develop at suboptimal temperature accumulate anthocyanins, which protect the leaves from photoinhibition without limiting photosynthesis (Pietrini et al., 2002). It is still not known which compound or reaction is primarily responsible for the improved chilling tolerance of the acclimated plants.

The role of the root system during chilling stress

When exposed to a sudden cold stress, maize seedlings exhibit symptoms of drought stress due to an imbalance between transpiration and water uptake (Aroca et al., 2003b). In particular, ineffective stomatal control is observed shortly after the onset of cold stress, which is the result of a decrease in the hydraulic conductance of the roots due to the greater viscosity of water at low temperature and to intrinsic characteristics of the root (Melkonian et al., 2004). The physiological activity of roots of chill-sensitive genotypes was marked by a stronger inhibition of water uptake and root respiration than chill-tolerant genotypes when exposed to chilling temperature (Sowinski and Małezewski, 1989). The chill-induced change in the water status induced the generation of abscisic acid (ABA), especially in chill-tolerant maize genotypes as was demonstrated under controlled conditions (Janowiak et al., 2002) as well as in the field following a cold spell (Janowiak et al., 2003). Furthermore, it was shown that the inhibition of ABA synthesis decreased tolerance to chilling, while the application of ABA increased it (Janowiak et al., 2002). Similar effects were observed when maize seedlings were pre-treated with drought; the drought-induced increase in the ABA content resulted in improved chilling tolerance (Aroca et al., 2003a;
Pérez de Juan et al., 1997). The way in which ABA affects chilling tolerance, however, remains to be elucidated.

When seedlings develop at low temperature the shoot/root ratio decreases, mainly due to a higher root dry matter at that particular growth stage (Richner et al., 1997). Furthermore, seedlings with a large amount of plant dry matter after growth at suboptimal temperature are characterized by a lower shoot/root ratio and a smaller leaf area/root length ratio (Hund et al., 2007). Although root architecture seems to be a constitutive trait, it correlates with chilling tolerance. The root system of chill-tolerant genotypes tends to be heterogeneous (lateral roots of the primary root are longer than the lateral roots of the seminal roots); the roots of chill-sensitive genotypes, however, exhibit a homogenous root system with primary and seminal roots of similar length (Hund et al., 2007). This indicates that at suboptimal growth temperature, too, the uptake of water or nutrients is disturbed. The uptake of phosphorus in particular seems to be important because of its low mobility; therefore, efficient root growth is essential to achieve adequate chilling tolerance (Chassot and Richner, 2002). The chill-induced decrease in phosphorus uptake may also explain why the leaves of maize seedlings under cold stress often turn purple, which is a characteristic of phosphorus deficiency.

The genetic basis of chilling tolerance

There is a large genetic variation in the chilling tolerance in maize. In particular, European flint and highland tropical material are characterized by greater chilling tolerance compared to dent material from the Corn Belt. Efficient breeding for improved chilling tolerance using these materials requires in-depth knowledge of the genetic basis of chilling tolerance. However, the inheritance of chilling tolerance is poorly understood. It is very difficult to deduce chilling tolerance of hybrid maize from the tolerance of its inbreds. The situation is complicated further by maternal effects in particular at early growth stages (Hodges et al., 1997).

**The genetic basis of chilling tolerance studied by QTL analyses**

QTL analysis is a useful tool for finding genomic regions responsible for chilling tolerance. Furthermore, it enables us to unravel the interaction of complex traits. Several QTLs for shoot fresh weight at early growth stages were identified under cool and moderately warm conditions (Presterl et al., 2007). Many of these QTLs are associated with leaf chlorosis but no obvious relationship was not found between the relative extent of the QTL effect and temperature conditions during growth. Focusing on photosynthesis-related traits, the main QTLs involved in the functioning of the photosynthetic apparatus were stable across cold environments (Fracheboud et al., 2004; Jompuk et al., 2005; Pimentel et al., 2005). Based on these QTL analyses, relationships were only frequently found between photosynthetic efficiency and greenness of the leaf. This indicates that the amount or the size of the photosynthetic units and their functioning are under different genetic control. The association between low chlorophyll content and a reduction in photosynthetic efficiency may reflect a disturbance of the assembly of the photosynthetic apparatus, induced by low growth temperature. Consequently, potential candidate genes, which are located near the major QTLs for chilling tolerance of photosynthesis, are genes involved in the assembly of the photosynthetic apparatus or genes of enzymes playing an important role in carbon assimilation (Jompuk et al., 2005).

A disadvantage of QTL experiments is that the results are valid only for the studied population. However, analogies between different populations were found when plants were raised under similar condi-

![Image](Figure 2: Chromosome 2 with complete QTL raw data for the photosystem II operating efficiency ($F_o'/F_m'$) determined in seedlings of three mapping populations (IBM302, R. Reimer, unpublished results; ETH-DL3 × ETH-DH7, Fracheboud et al. (2004); ETH-EH3 × ETH-EL1, J. Leipner, unpublished results) grown at suboptimal temperature. The colors range from blue (low LOD score) to red (high LOD score). Homologous markers between populations are connected by lines. Positions of markers are indicated, which were found in the vicinity of QTLs for fresh matter yield (dupssr21) and frost damage (bnlg1184) in field-grown seedlings (Presterl et al., 2007) and for $F_o'/F_m'$ in seedlings grown at suboptimal temperature (umc98) (Fracheboud et al., 2002).)
tions and when the traits characterizing the photosynthetic apparatus were considered, indicating to some extent a common genetic basis of chilling tolerance of photosynthesis. For example, on the short arm of chromosome 2, near the SSR marker phi109642, a common QTL was found for leaf greenness, carbon exchange rate and the photosystem II operating efficiency \((F_o/F_m')\) in seedlings of the ETH-DL3 × ETH-DH7 population grown under suboptimal temperature in growth chambers as well as in the field (Fracheboud et al., 2004; Jompuk et al., 2005). QTLs for \(F_o/F_m'\) were detected at the same position in two other mapping populations, derived from the hybrids B73 × Mo17 and ETH-EH3 × ETH-EL1, when both were grown at suboptimal temperature (Figure 2). In this region but at some distance from the former QTLs, QTLs were found for the carbon exchange rate and \(F_o/F_m'\) (Fracheboud et al., 2002) as well as for frost damage and fresh weight of seedlings grown under the temperate conditions of central Europe (Presterl et al., 2007).

**Molecular basis of chilling tolerance**

Much of our knowledge of the molecular response during response to cold and of cold-acclimation is based on studies of *Arabidopsis thaliana*. Although some of this knowledge aids the better understanding of the chilling tolerance/sensitivity of maize, there are large phylogenetic, morpho-physiological and eco-physiological differences between *Arabidopsis* and maize, which requires a detailed molecular biological analysis of maize as well. However, our knowledge of the molecular response of maize to chilling stress is rather rudimentary. Moreover, most of the information about cold-induced genes was gathered after a rapid decrease in temperature (usually from 25 to 5 °C), which poorly reflects the situation in the field. This type of experimentation is necessary, however, to unravel step by step the molecular response of maize to low temperature.

The increase in cytoplasmic \(\text{Ca}^{2+}\) seems to be an early response to exposure of plants to low temperature and may be triggered by cold-induced rigidification of the plasma membrane. In maize, the gene *ZmCDPK1*, coding a calcium-dependent protein kinase, is induced at low temperature (Berberich and Kusano, 1997), indicating that \(\text{Ca}^{2+}\) signals are important in the response of maize to cold stress. Time course experiments revealed that induction of *ZmCDPK1* precedes that of *mlip15*, another gene induced by cold, which codes for a DNA-binding protein of the basic region/leucine zipper (bZIP) type (Kusano et al., 1995). Therefore, *ZmCDPK1* might be located upstream of *mlip15* in the cold-stress signaling pathway. The presence of *mlip15* transcripts in senescent maize leaves and the cold induction of *ZmMAPK5*, transcribing a mitogen-activated protein kinase (MAPK) that is known to be involved in senescence, indicate that cold promote programmed cell death (Berberich et al., 1999).

As well as the induction of the bZIP transcription factor MLIP15, cold induces the transcription of ERF/AP2 type transcription factors in maize. One of these transcription factors, *ZmDREB1A*, was expressed during cold stress but almost no transcripts were found after ABA application (Qin et al., 2004), indicating an ABA independent pathway. Other genes for transcription factors that are induced in maize at low temperature, namely maize *DBF1* (Zheng et al., 2006) and *ZmDREB2A* (Nguyen, 2005; Qin et al., 2007), seem to be part of the ABA-dependent signaling since they are also induced by drought, salt or ABA application.

Other cold-induced genes are involved in DNA methylation (*ZmMET1*) (Steward et al., 2000) or in phytohormone metabolism: e.g. genes for zeatin O-glycosyltransferase (Li et al., 2000) and 12-oxophytodienoic acid reductase (Nguyen, 2005; Zheng et al., 2006). The role of the fatty acid composition during acclimation to cold was supported by the differential expression of the \(\omega-3\) fatty acid desaturase genes, *FAD7* and *FAD8*, upon exposure to cold temperature (Berberich et al., 1998). Furthermore, the cold-induced expression of the *cat3* gene coding a catalase (Anderson et al., 1994) and of several genes of the anthocyanin pathway (Christie et al., 1991) underlines the importance of ROS defense and light protection, respectively, in the acclimation of maize seedlings to chilling.

Few attempts were made to increase the chilling tolerance of maize seedlings by transformation. Seedling were produced, which overexpressed Mn-superoxide dismutase (Kingston-Smith and Foyer, 2000b; Van Breusegem et al., 1999). Although changes in the antioxidative defense and in tolerance to methyl viologen-induced oxidative stress occurred, there was not a significant improvement of chilling tolerance. Similarly, the expression of the cold-tolerant pyruvate orthophosphate dikinase from *Flaveria brownii* in maize had very little effect on the chilling tolerance of photosynthesis (Ohta et al., 2006). Enhanced tolerance to freezing was achieved by transforming maize with *NPK1*, a tobacco mitogen-activated protein kinase kinase kinase, which is assumed to be involved in an \(\text{H}_2\text{O}_2\) signaling pathway (Shou et al., 2004). The plants in this study were characterized by a higher level of sugar and withstood a lower freezing temperature compared to the control plants. The application of glycinebetaine, which maize can synthesize only in small amounts if at all, improves chilling tolerance (Chen et al., 2000). Based on this finding Quan et al. (2004) transformed maize with the *betA* gene encoding a choline dehydrogenase from *Escherichia coli*. Transformants showed a higher level of glycinebetaine, which was related to better shoot growth, less cell injury, in-
creased survival, a decrease in photoinhibition and higher photosynthetic activity at low temperature. Although the superiority of these transgenic plants has yet to be proven under conditions in the field, the results are very promising for a successful improvement of chilling tolerance by means of transformation.

Conclusions

Current knowledge of the complex physiological and molecular biological effects of low temperature on maize seedlings is based on extensive studies, most of which were performed in controlled environment. The seedling’s response to low temperature was determined and genotypes with contrasting tolerance to cold stress were compared. These experiments have shown that, for a few days, maize seedlings can withstand temperatures as low as 5 °C without showing signs of lasting damage, even though the effect on photosynthesis is considerable under such conditions. Tissue that develops under such low temperature, however, is irreparably damaged. The development of the photosynthetic machinery seems to play a crucial role in this effect. Nevertheless, the primary cause and site of this chilling-induced damage are still unclear. The recent development of molecular and quantitative genetics enables the detailed study of complex physiological processes. As well as a better understanding of the basic mechanisms of chilling tolerance, this approach may help to find molecular markers for marker-assisted breeding of stress-tolerant genotypes. The molecular biological studies will be challenging, because a main focus should be on the effect of chilling on developmental processes.

Goals and objectives

Much knowledge has been gained about the physiological response of maize to low temperature and how it acclimates to suboptimal temperature; our own earlier findings concerning this topic are summarized in Chapter 2. However, the key factor which differentiates chilling-tolerant from chilling-sensitive genotypes still remains open. Furthermore, the molecular response and the genetic background of the response to low temperature are largely unknown. Therefore, the overall goals were to gain a better understanding of the genetics of chilling tolerance by quantitative genetics and to get information about the molecular mechanisms of chilling tolerance by studying the expression of cold-induced genes.

In order to achieve these goals the following objectives were envisaged:

to develop a method, which allows to efficiently discriminate maize material of contrasting chilling tolerance, and to use this method to generate inbred lines with high and low chilling tolerance suitable for the production of a segregating population (Chapter 3)
to map quantitative trait loci (QTLs) for chilling tolerance of photosynthesis in this segregating population grown under controlled conditions (Chapter 4)
to verify the QTLs under field conditions (Chapter 5) and to study their importance for yield-related traits (Chapter 6)
to examine the importance of the excitation pressure for the expression of QTLs associated with chilling tolerance of photosynthesis (Chapter 7)
to investigate the effect of the temperature at night on the expression of QTLs for photosynthesis-related traits in an alternative population, which would also allow to verify the previously identified QTLs in a different genetic background and/or give the possibility to identify other genomic regions involved in the chilling tolerance of photosynthesis (Chapter 8)
to use the QTL analysis to study the interaction among morpho-physiological and biochemical traits; in particular, by examining the importance of the C₄ cycle enzymes and antioxidants in the chilling tolerance of photosynthesis (Chapter 9) as well as the effect of chilling tolerance of photosynthesis on the specific leaf area (SLA) (Chapter 10).
to identify new cold-induced genes in maize seedling and to examine their role in the general stress response (Chapter 11)
to characterize a new highly expressed cold-induced gene and to analyze its potential function in the response to low temperature and other abiotic stress factors (Chapter 12).
Review of previous research

Acclimation of defense systems in maize seedlings to chilling stress

One of the major effects of suboptimal growth temperature is its impact on the photosynthetic apparatus, which is manifested in a decrease in photosynthetic capacity and efficiency (Haldimann et al., 1996; Massacci et al., 1995; Nie et al., 1992; Verheul et al., 1995). The decline in photosynthetic capacity is accompanied by a lower activity of certain enzymes of the Benson-Calvin cycle (Stamp, 1987b) and an impairment of chloroplast development (Nie and Baker, 1990). Whereas, the depressed photosynthetic efficiency seems to be based on a zeaxanthin-related quenching of absorbed excitation at photosystem II (PSII) (Fryer et al., 1995). Pigment analyses disclosed an accumulation of zeaxanthin in maize leaves that developed at suboptimal temperature, especially under high light intensity (Haldimann et al., 1995). In light of the fact that maize leaves which develop at suboptimal temperature recover faster from photoinhibition of photosynthesis, due perhaps to their higher capacity for energy dissipation (Haldimann et al., 1996), we investigated their ability to resist photooxidative stress (Leipner et al., 1997). Maize leaf segments of seedlings grown at suboptimal (15 °C) or optimal temperature (25 °C) were subjected to high irradiance (1000 µmol m⁻² s⁻¹) at chilling temperature (5 °C), and the changes in pigment and antioxidant contents were monitored. During this stress, the degradation of the endogenous antioxidants ascorbate, glutathione and α-tocopherol was delayed and less pronounced in 15 °C leaves. Similarly, the decline in chlorophyll a, chlorophyll b, β-carotene and lutein was slower throughout the stress period. Faster development and a higher level of non-photochemical quenching (NPQ) of chlorophyll fluorescence, which was related to a stronger de-epoxidation of the larger xanthophyll cycle pool in 15 °C leaves, could act as a defense mechanism to reduce the formation of reactive oxygen species during severe chilling. Furthermore, plants grown at suboptimal temperature exhibited a higher amount of the antioxidants glutathione and α-tocopherol. The higher α-tocopherol content in leaves (double based on leaf area; four-fold higher based on chlorophyll content) which developed at suboptimal temperature may play an especially important role in the stabilization of the thylakoid membrane and thus prevent lipid peroxidation. The results suggest that growth at suboptimal temperature diminishes oxidative damage during chilling stress under high light intensity. More efficient dissipation of excess absorbed light energy, due to a higher de-epoxidation state of the larger xanthophyll cycle pool, seemed to be the major defense mechanism against the formation of reactive oxygen in leaves that developed at suboptimal temperature. Furthermore, a higher amount of glutathione and especially of α-tocopherol may minimize the harmful effects of the reactive oxygen species.

Studies on field-grown maize (Fryer et al., 1998) and on cucumber leaves (Terashima et al., 1998) consider the formation of superoxide radicals at photosystem I (PSI) to be the primary cause of chilling-induced damage by light to the photosynthetic apparatus. However, a chilling-induced consumption of antioxidants was observed only when severe chilling stress was combined with high light intensity stress (Leipner et al., 1997; Wise and Naylor, 1987).
Therefore, the question arose whether chilling at natural light conditions induces an oxidative stress which cannot be sufficiently scavenged by the antioxidative systems in maize leaves. Thus, maize seedlings were grown at optimal (25 °C) and suboptimal (15 °C) temperature and then exposed to severe chilling temperature (6 °C) at their growth light intensity (450 µmol m⁻² s⁻¹) for 4 days (Leipner et al., 2000a). Photosynthetic parameters, hydrogen peroxide, antioxidant contents and activity of scavenging enzymes were investigated before, during and after chilling stress. This stress caused a stronger reduction in photosynthetic activity, maximum quantum efficiency of PSII primary photochemistry (F_/F_m) and catalase activity in plants which had been grown at 25 °C rather than at 15 °C. Maize plants grown at suboptimal temperature de-epoxidized their xanthophyll cycle pool to a greater extent and exhibited a faster recovery from chilling stress than plants which had not been acclimated to chilling. Antioxidant content, activity of scavenging enzymes (with the exception of catalase), hydrogen peroxide formation and the size of the xanthophyll cycle pool were hardly affected by chilling stress. However, chilling induced a temporary increase in the glutathione content and triggered the synthesis of α-tocopherol during the phase of recovery at 25 °C. The results indicated that leaves respond to chilling stress by down-regulation of PSII accompanied by de-epoxidation of the xanthophyll cycle pool, probably to prevent enhanced formation of superoxide radicals at PSI, and consequently other reactive oxygen species. The discrepancy between the recovery of F_/F_m and photosynthesis as well as the increase in the contents of α-tocopherol and glutathione after transfer to optimal temperature indicated that re-warming might be a critical phase due to a disturbance in photosynthesis. The transient increase in H₂O₂ formation in 15 °C plants that recover at 15 °C after chilling stress is a further indication of transient oxidative stress associated with the onset of re-warming. Nevertheless, the increase in temperature after chilling stress promotes further adaptation processes that might diminish chill-induced damage during a subsequent period of chilling temperature.

Since maize leaves grown at suboptimal temperature are characterized by an increase in the concentration of certain antioxidants (Kingston-Smith et al., 1999; Leipner et al., 1997), we suggested that the higher antioxidative and dissipative ability may be the cause of the lower susceptibility of the chilling-acclimated leaves to photooxidative stress. To determine whether better tolerance to low-temperature stress is due mainly to the antioxidants or to dissipative mechanisms, the ability of leaves with an artificially increased content of ascorbate to resist photoinhibition and photooxidative stress was investigated (Leipner et al., 2000b). Infiltrating detached maize leaves with L-galactono-1,4-lactone (L-GAL), which is the precursor of ascorbate in its synthesis (Wheeler et al., 1998), resulted in a four-fold increase in the content of leaf ascorbate. Upon exposure to high irradiance (1000 µmol m⁻² s⁻¹) at 5 °C, L-GAL leaves de-epoxidized the xanthophyll-cycle pigments faster than the control leaves. The maximal ratio of de-epoxidized xanthophyll-cycle pigments to the whole xanthophyll-cycle pool was the same in both leaf types. The elevated ascorbate content, together with the faster violaxanthin de-epoxidation, did not affect the degree of photoinhibition and the kinetics of the recovery from photoinhibition, assayed by monitoring the maximum quantum efficiency of PSII primary photochemistry (F_/F_m). Under the experimental conditions, the thermal energy dissipation seems to be zeaxanthin-independent since, in contrast to the de-epoxidation, the decrease in the efficiency of excitation-energy capture by open PSII reaction centers (F_/F_m) during the high-irradiance treatment at low temperature showed the same kinetic in both leaf types. This was also observed for the recovery of the maximal fluorescence after stress. Furthermore, the elevated ascorbate content did not diminish the degradation of pigments or α-tocopherol when leaves were exposed for up to 24 h to high irradiance at low temperature. Moreover, a higher content of ascorbate appeared to increase the requirement for reduced glutathione. Overall, the de-epoxidation of violaxanthin via antheraxanthin to zeaxanthin was faster in L-GAL-treated leaves probably due to their higher ascorbate content. However, the results indicate that neither ascorbate nor the ascorbate-dependent increase in the de-epoxidation rate can markedly protect maize leaves against the photoinhibition of photosynthesis or photooxidative damage to the photosynthetic apparatus. Adding more ascorbate even appeared to increase the requirement for reduced glutathione (GSH) and did not seem to improve the recycling of α-tocopherol in vivo. Therefore, we concluded that, with respect to acclimation to low temperature, an increase in the ascorbate contents might not be a reason for the superior ability of chilling-acclimated leaves to diminish photooxidative damage. Rather, the presence of zeaxanthin before the onset of photoinhibitory stress, which is the case in maize leaves acclimated to suboptimal temperature (Haldimann et al., 1995), might cause the improved ability of chilling-acclimated maize plants to withstand low-temperature induced photooxidative stress.

During early growth of maize in the field, seedlings especially of chilling-sensitive genotypes often suffer from low temperature resulting in reduced growth (Verheul et al., 1996). Periods of chilling temperature seem to be accompanied with an appearance of an alternative electron sink, probably the Mehler reaction (Fryer et al., 1998). Therefore we hypothesized...
that, under chilling conditions, the Mehler reaction is more enhanced in chilling-sensitive than in chilling-tolerant genotypes. To prove this, the $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ ratio and the antioxidative capacity were measured in a chilling-tolerant and a chilling-sensitive genotype (Leipner et al., 1999a). Previous comparative studies of chilling stress early in the growing season were conducted with maize plants of different physiological age (Andrews et al., 1995; Fracheboud and Haldimann, 1994; Fryer et al., 1998). To separate an ontogenetic influence from environmental effects, maize was sown in three subsequent weeks. Thus, plants sown early were exposed to chilling conditions, whereas plants sown later developed under conditions more favorable for growth. Measurements of the quantum efficiency of CO$_2$ fixation ($\Phi_{\text{CO}_2}$) and of the photosystem II ($F'_q/F'_v$; in this context also termed $\Phi_{\text{PSII}}$) were made simultaneously on the third fully expanded leaves. The activity of scavenging enzymes and the content of pigments and antioxidants were also determined. Leaves that developed under chilling conditions showed typical chill-induced alterations, namely low photosynthetic capacity and efficiency, reduction in the pigment contents and a decrease in catalase activity. These alterations were more pronounced in the chilling-sensitive than in the chilling-tolerant genotype. Determining the $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ ratio indicated that it was very unlikely that alternative electron sinks, such as the Mehler reaction, were activated to a significant extent in either of the genotypes. This was supported by the measurements of the activity of enzymes involved in the Mehler ascorbate peroxidase reaction. However, a comparison of the genotypes showed that chilling tolerance might be correlated with an increase in the $\alpha$-tocopherol and glutathione contents as well as in the activity of glutathione reductase. In conclusion, maize plants grown in the early season in the field showed typical chill-induced reduction of their photosynthetic performance. However, there was no evidence of oxidative stress due to an enhanced Mehler reaction, since the ratio of $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ was usually close to the expected 12:1 relationship for maize leaves (Edwards and Baker, 1993). Furthermore, neither an accumulation of antioxidants, nor an increase in the activity of scavenging enzymes, which would in turn indicate an increase in the formation of ROS, were found in plants with low photosynthetic performance. Comparing genotypes of different chilling tolerance showed that chilling tolerance might be correlated with a constitutive increase in antioxidative capacity, thus implying the presence of ROS under chilling conditions. However, it could not be clearly determined whether these alterations in the contents of antioxidants brought about chilling tolerance.

Taking together, alterations in scavenging systems seem to support the more efficient dissipation of the excess of absorbed light energy in leaves that developed at suboptimal temperature. This seems to pre-

![Figure 1](image-url)

**Figure 1**: Schematic representation of the effect of low temperature on the photosynthetic apparatus and scavenging systems of maize leaves grown at optimal (A) and suboptimal temperature (B). Chilling led to reduced carbon fixation and to enhanced dissipation of the excess of absorbed light energy as heat. Excess energy can be used by the Mehler reaction too. If these mechanisms are insufficient to consume the absorbed light energy then $^1$O$_2$ is generated. Scavenging systems (sponges) remove ROS until their capacity is depleted. GSH (oil-can) is essential for optimal carbon fixation.
vent chill-induced damage and to support a fast recovery from chilling stress. Thereby, α-tocopherol mainly protects the photosynthetic apparatus itself, whereas glutathione, in conjunction with glutathione reductase, protects enzymes which are involved in carbon fixation. These effects of low temperature on the photosynthetic apparatus and the defense systems are summarized in Figure 1. Nevertheless, the response of the scavenging systems to low temperature is rather slow. Together with the pool size of the xanthophyll cycle, they represent the long-term adaptation of the plant. However, non-photochemical quenching together with the de-epoxidation state of the xanthophyll cycle pool are more flexible and seem to be major defense mechanisms that can be regulated within minutes to hours, even at low temperature.

Maize genetic resources from Mexican highland are known to be a potential source for improved chilling tolerance (Brooking, 1990; Stamp, 1985). This geographic region is characterized by average temperatures of about 15 °C during the growing season with minimum temperature frequently below 10 °C. However, less attention has been paid to the effects of excess heat, although it is suspected that low yield potentials of maize containing tropical highland germplasm might be due to heat sensitivity (Hardacre and Eagles, 1989). We studied genetic resources from Mexican highland as a model source for an improved early seedling vigor at low temperature (Leipner et al., 1999b). In order to understand more about a possible high temperature sensitivity of this material, two European and two part-exotic highland cultivars were grown at 16 °C, 25 °C and 33 °C until the sixth leaf stage. High growth temperature and, to a greater extent low growth temperature, reduced the total leaf area. However, part-exotic cultivars were much more affected by high growth temperature than the European cultivars. Photosynthesis-related traits were measured on the fully developed fifth leaf. The rate of photosynthesis per unit leaf area was reduced by low growth temperature, especially in the European cultivars. The part-exotic cultivars were less affected; this was partly due to their higher chlorophyll content, higher PSII operating efficiency ($F_{v}/F_{m'}$) and lower non-photochemical quenching. An increased stomatal resistance at low growth temperature reflected also the genotypic differences in photosynthesis rates. In contrast to photosynthesis and $F_{v}/F_{m'}$, the decrease of $F_{v}/F_{m}$ was induced by both low and high growth temperature. In the part-exotic cultivars, this decrease of $F_{v}/F_{m}$ was smaller than in the European cultivars at low growth temperature, but it was more pronounced at high growth temperature. It was concluded that photosynthesis-related traits are more chilling stable in Mexican highland material. However, this is related to a reduced fitness at high temperature which is a major obstacle for a fast integration in European breeding material. The good photosynthetic performance under suboptimal temperature would be very valuable in breeding programs if the linkage to a low leaf area ratio (LAR) and reduced growth vigor at high temperature could be broken.

**Acclimation of maize seedlings to heat stress**

Chilling and heat are opposite stresses on the temperature scale. With climate change chilling and heat tolerance may become prerequisites for yield consistent maize germplasm in continental climates. To get new insights into the mode of acclimation to heat stress, we addressed the question whether the primary target of heat-induced perturbation of photosynthesis is identical in heat-acclimated and non-acclimated maize plants (Sinsawat et al., 2004). To investigate this, the effect of high growth temperature (41 °C versus 25 °C) on the heat tolerance of the photosynthetic apparatus in maize leaves was studied by means of chlorophyll fluorescence measurements and photosynthetic oxygen evolution. Heat stress treatment was conducted in the dark to prevent heat-induced photoinhibition as well as leaf cooling due to transpiration. Exposure of plants grown at 25 °C to 35 °C for 20 minutes in the dark led to a transient inhibition of the rate of photosynthesis by about 50%, which recovered within 4 hours. Treatments at temperatures above 45 °C led to permanent damage; the plants did not recover within 96 hours. In contrast, no damage occurred to plants grown at 41 °C after exposure to temperatures up to 50 °C. Improved thermo-tolerance as a result of high growth temperature was not related to the presence of zeaxanthin, since it did not accumulate in heat-stressed leaves in the dark. In plants grown at 25 °C, measurements of the PSII operating efficiency ($F_{v}/F_{m'}$) in leaves infiltrated with methylviologen (MV) indicated the presence of a heat-sensitive component of the photosynthetic apparatus, located downstream of photosystem II and before the carbon cycle.
Chapter 3

Chlorophyll fluorescence as a selection tool for chilling tolerance of photosynthesis in maize seedlings*

The possibility of using quenching analysis of chlorophyll a fluorescence as a selection tool for improving the chilling tolerance of maize was investigated in six genotypes differing greatly in the ability to develop a competent photosynthetic apparatus at low temperature. Upon gradual cooling measurements of the photosystem II operating efficiency ($F_{q'}/F_{m'}$) indicated that leaves of tolerant genotypes that developed at suboptimal temperature (15 °C) maintained higher rates of electron transport than leaves of sensitive genotypes. This difference was largely due to the ability of the tolerant plants to keep higher efficiency of excitation energy capture by open photosystem II reaction centers ($F_{v'}/F_{m'}$). The absence of genotypic differences in leaves that developed at optimal temperature indicates that the trait is not expressed constitutively, but relies on adaptation mechanisms. Furthermore, the genotypic difference was not expressed under increasing illumination at 15 °C and 25 °C suggesting that the trait is also low-temperature-specific and is not expressed solely in response to increasing excess light energy. Applying the method to flint and dent breeding population led to a substantial increase (up to 31%) in the photosynthetic capacity of hybrids between selected F3 inbreeding families grown at suboptimal temperature, demonstrating that the method is an efficient selection tool for improving the chilling tolerance of maize through breeding.

Introduction

It is necessary to improve the adaptation of maize to low temperature, because over the past 50 years, the cultivation of maize has been extended to areas in cooler regions. It has become a major crop in northern regions where its high temperature requirement is not always fulfilled. Suboptimal temperature during spring results in decreased productivity (Carr and Hough, 1978) and poor yield stability (Stamp, 1986). Amongst the various effects of low temperature on the physiology of maize (Stamp, 1984), the high susceptibility of the photosynthetic apparatus to low temperature is considered to be of particular importance (Baker, 1994; Hayden and Baker, 1989). The photosynthetic apparatus of maize is known to be highly sensitive to low temperature-induced photoinhibition (Long et al., 1983; Nie et al., 1992). Leaves that develop at a temperature of 15 °C or below are also characterized by a very low photosynthetic capacity (Haldimann et al., 1996; Nie et al., 1992), altered leaf pigment composition (Haldimann et al., 1995; Haldimann, 1998) and impaired chloroplast development (Robertson et al., 1993). Improved chilling tolerance of the photosynthetic apparatus of maize may, therefore, contribute substantially to improving the performance of the crop in temperate regions by increasing early vigor and extending the culture period.

In the past 15 years in vivo chlorophyll fluorescence measurements are commonly used to study the functioning of the photosynthetic apparatus. In particular, quenching analysis using the saturation pulse technique (Schreiber et al., 1986) provides semiquantitative information about photochemistry in intact leaves. This method has been used extensively to investigate the response of plants to environmental stress, including the effects of low temperature on the photosynthetic apparatus of maize both in a controlled environment (Haldimann et al., 1996; Havaux, 1987) and in the field (Andrews et al., 1995). The use of chlorophyll fluorescence measurements as a screening method for chilling tolerance has already been investigated in experiments in which genotypes were compared (Dolstra et al., 1994; Havaux, 1987; Hetherington et al., 1983; Schapendonk et al., 1989). The genotypic variability of plants developed at suboptimal temperature has, however, not been investigated. Results of several studies indicate that the photosynthetic apparatus of maize can adapt to suboptimal growth temperature and become more tolerant to severe chilling stress. It has been shown, for example, that such leaves recover faster from photoinhibition (Haldimann et al., 1996), are more tolerant to chilling-induced photooxidation and contain greater amounts of certain leaf

* based on the following publication:
antioxidants (Leipner et al., 1997). Thus, it is likely that some of the genotypic differences related to low temperature tolerance may be detectable only in leaves that develop at low temperature.

The present study analyses the temperature response of chlorophyll fluorescence quenching in maize leaves developed at optimal and suboptimal temperature. Genotypes of different origin were compared to determine the conditions necessary to apply chlorophyll fluorescence analysis as a selection method to improve chilling tolerance of photosynthesis. The validity of the method was demonstrated by the improvement in the photosynthetic capacity of hybrids between F₃ inbreding families grown at suboptimal temperature.

**Materials and methods**

**Plant material**

Six *Zea mays* L. lines used in the experiments consisted of two chilling-tolerant inbreds of European origin, Z7 and Z15, the hybrid between these two tolerant lines (Z7 × Z15), two chilling-sensitive inbreds of tropical origin, Penjalina (PENJ) and CM109, and the hybrid between the two sensitive lines (PENJ × CM109). The seedlings were grown in growth chambers (Conviron PGW36, Winnipeg, Canada) in 1.0 l pots in a soil/sand mixture (10:1, v/v) under a 12 h photoperiod (450 µmol m⁻² s⁻¹) at a relative humidity of 60/70% (day/night). The plants were first grown at 25/22 °C (day/night) for 5 d and then grown at 25/22 °C or at 15/13 °C until full development of the third leaf. The plants were watered and fertilized with half-strength Hoagland nutrient solution as required. All measurements were performed on the fully expanded third leaves.

**Measurements of photosynthesis**

O₂ evolution was measured on leaf segments with a leaf disc electrode unit (LD2/Z, Hansatech, King’s Lynn, UK) in 5% CO₂ at 15 °C or 25 °C. Light was provided by a Bjorkman lamp (LS2, Hansatech, King’s Lynn, UK). The rate of photosynthetic O₂ evolution was obtained by subtracting the rate of dark respiration from the oxygen evolution rate in the light.

The net rate of CO₂ assimilation was measured on the middle part of fully developed third leaves using a portable LI-COR 6200 apparatus (LI-COR, Lincoln, USA) under growing conditions.

**Determination of chlorophyll content**

The content of chlorophyll *a+b* was determined from 1 cm diameter leaf discs extracted with 2 ml 80% acetone (Arnon, 1949).

**Chlorophyll fluorescence measurements**

Chlorophyll a fluorescence was recorded with a pulse amplitude modulation fluorometer (PAM-2000, Walz, Effeltrich, Germany). The photochemical quenching factor (**Fₚ'/Fₚ**), the efficiency of excitation energy capture by open photosystem II (PSII) reaction centers (**Fₚ'/Fₚ**) and the PSII operating efficiency (**Fₚ'/Fₚ'**') were determined (Genty et al., 1989). **Fₚ'**, which was used for the determination of **Fₚ'/Fₚ'** and **Fₚ'/Fₚ'**' (Bilger and Schreiber, 1986), was measured after switching off the actinic light and simultaneously applying 3 s of far red light (735 nm, 15 W m⁻²).

**Response of chlorophyll fluorescence parameters to decreasing temperature**

Intact plants were dark-adapted in a growth chamber at 25 °C for 15 min. The middle part of the third leaf was fixed on a leaf clip (2030-b, Walz, Effeltrich, Germany) which allows to record the temperature of the leaf. The maximum quantum efficiency of PSII primary photochemistry (**Fₚ'/Fₚ**) was then determined by application of a 1 s saturation flash (> 10,000 µmol m⁻² s⁻¹). The leaves were then exposed to actinic illumination (125 µmol m⁻² s⁻¹) of red light (655 nm) provided by the light-emitting diode array of the PAM fluorometer. After 15 min adaptation to light, the plant was cooled gradually from 25 °C to 2.5 °C at a rate of 0.17 °C min⁻¹ while saturation flashes were applied every 75 s. The recorded data for each leaf were pooled by 1 °C intervals.

**Response of Fₚ'/Fₚ' to increasing light intensity**

Intact plants adapted to the dark (15 min at room temperature) were placed in a growth chamber at 25, 15 or 6 °C. The middle part of the third leaf was fixed on the PAM leaf clip and the plant was left in the dark until the leaf temperature and ambient temperature were the same. After **Fₚ'/Fₚ'** determination, the leaf was exposed to the lowest actinic illumination (50 µmol m⁻² s⁻¹) for 20 min before **Fₚ'/Fₚ'** was determined by five successive saturation flashes at 1 min intervals. Thereafter, the leaf was adapted for 10 min to each light intensity (up to 1000 µmol m⁻² s⁻¹) before determination of **Fₚ'/Fₚ'**. Five successive measurements were made to test if **Fₚ'/Fₚ'** had reached a steady-state value since the last change of actinic light intensity. The fact that **Fₚ'/Fₚ'** did not change significantly between the first and the fifth measurement (data not shown) indicated that 10 min adaptation was sufficient to reach a stable **Fₚ'/Fₚ'** value under all light and temperature conditions tested. Actinic light was provided by a KL1500 lamp (Schott, Mainz, Germany) through an optic fiber. For each light intensity, **Fₚ'/Fₚ'** was estimated from the average of the five measurements.
Maize families selected from breeding populations for high $F_q/F_m$ at low temperature

A Swiss dent and a Swiss flint corn type breeding population were used for the selection procedure. First, 40 seeds of each population were sown in the field and plants of each population were intercrossed yielding 31 ears of flint and 38 ears of dent. Twelve plants of each of these single ear progenies were grown in a growth chamber at suboptimal temperature as described for the model genotypes. The $F_q/F_m$ was measured on the third fully expanded leaf at 6 °C and an actinic illumination 60 µmol m$^{-2}$ s$^{-1}$. The average value of the measurements of the 12 plants enabled us to determine the eight families in each population with the highest $F_q/F_m$. These families were used for further selection. Forty plants of each of these selected F1 families were grown and analyzed under the same conditions in order to select the best four individuals in each family. These plants were transplanted to the field and self-pollinated, yielding one to four F2 ears, depending on the family. Fifteen plants of each progeny were then tested as described above to determine the best four F2 families in each population and the best progeny within these families. Forty plants of each of the selected F2 families were then tested to determine the best five individuals. These were transferred to the field and self-pollinated. Ten plants of each F3 ear were then tested to determine the best progeny of each family. These were denominated H1, H2, H3 and H4, according to their average $F_q/F_m$ value (in decreasing order). Forty plants from each of these selected F3 families were then analyzed as described above to determine the best five individuals, which were then transferred to the field. Hybrids between the different families were produced to compare with the original breeding populations. Hybrids were preferred to inbreds to avoid the undesirable effects of inbreeding depression.

Results

The photosynthesis of all lines was strongly affected by temperature (Figure 1). The rate of photosynthetic oxygen evolution in leaves developed at 25 °C was greatly reduced by decreasing the measuring temperature from 25 °C to 15 °C. There was

\[\text{Figure 1: The effect of temperature and light intensity on the photosynthetic oxygen evolution of the third leaves of chilling-tolerant (closed symbols) and chilling-sensitive (open symbols) maize genotypes developed at 25 °C (A, C) and 15 °C (B, D) and measured at 25 °C (A, B) or 15 °C (C, D). (●) Z7, (▲) Z15; (▼) Z7 × Z15; (○) PENJ; (△) CM109; (▽) PENJ × CM109. Values are means (±SD) of five plants. Stars indicate light intensities where pooled tolerant genotypes differed from pooled sensitive genotypes in a t-test comparison with } P < 0.001.\]
Table 1: The maximum quantum efficiency of PSII primary photochemistry \( (F_v/F_m) \) and the chlorophyll \( a+b \) content in maize leaves developed at 25 °C or 15 °C.

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<tr>
<td>Z7</td>
<td>( F_v/F_m )</td>
<td>0.780 ± 0.010</td>
<td>0.668 ± 0.014</td>
<td>491 ± 43</td>
<td>319 ± 48</td>
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<tr>
<td>Z15</td>
<td>( F_v/F_m )</td>
<td>0.785 ± 0.007</td>
<td>0.709 ± 0.016</td>
<td>598 ± 49</td>
<td>512 ± 65</td>
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<tr>
<td>Z7 × Z15</td>
<td>( F_v/F_m )</td>
<td>0.794 ± 0.011</td>
<td>0.713 ± 0.017</td>
<td>516 ± 22</td>
<td>386 ± 20</td>
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<tr>
<td>PENJ</td>
<td>( F_v/F_m )</td>
<td>0.780 ± 0.006</td>
<td>0.705 ± 0.020</td>
<td>351 ± 49</td>
<td>171 ± 39</td>
</tr>
<tr>
<td>CM109</td>
<td>( F_v/F_m )</td>
<td>0.794 ± 0.004</td>
<td>0.708 ± 0.015</td>
<td>464 ± 39</td>
<td>189 ± 51</td>
</tr>
<tr>
<td>PENJ × CM109</td>
<td>( F_v/F_m )</td>
<td>0.780 ± 0.008</td>
<td>0.722 ± 0.020</td>
<td>483 ± 60</td>
<td>195 ± 51</td>
</tr>
<tr>
<td>CT</td>
<td>Chl ( a+b ) (µmol m(^{-2}))</td>
<td>0.783 ± 0.010</td>
<td>0.696 ± 0.026</td>
<td>556 ± 83</td>
<td>399 ± 99</td>
</tr>
<tr>
<td>CS</td>
<td>Chl ( a+b ) (µmol m(^{-2}))</td>
<td>0.785 ± 0.009 NS</td>
<td>0.712 ± 0.020 NS</td>
<td>422 ± 83 ***</td>
<td>185 ± 42 ***</td>
</tr>
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</table>

CT: pooled tolerant genotypes; CS: pooled sensitive genotypes. Values are means \( (±SD) \) of five plants. Stars indicate t-test comparison between CT and CS for a given growth temperature. ***: \( P < 0.001 \), NS not significant at \( P < 0.05 \).

no clear genotypic difference in leaves grown at 25 °C, with the exception that the chilling-sensitive line, Penjalinan, had a slightly lower photosynthetic capacity than the other lines when the measurements were performed at 15 °C (Figure 1C). Photosynthetic capacity was lowest in leaves developed and measured at 15 °C (Figure 1D). Under these conditions, the photosynthetic capacity of the three tolerant genotypes was significantly higher than the photosynthetic capacity of the three sensitive genotypes. Furthermore, when the measurement temperature was increased to 25 °C the photosynthetic capacity of two of the tolerant genotypes increased, in contrast to the sensitive genotypes (Figure 1B). Tolerant genotypes were also characterized by a higher chlorophyll content than sensitive genotypes at both growth temperatures (Table 1), but the reduction in the chlorophyll content in response to low temperature was more pronounced in the sensitive genotypes (-54% on average) than in the tolerant genotypes (-24% on average). While the genotypic difference in the chlorophyll content correlated with the difference in the photosynthetic capacity in 15 °C leaves, this was not the case in 25 °C leaves (Figure 1). Growth at low temperature also led to a slight, but significant decrease in the maximum quantum efficiency of PSII primary photochemistry \( (F_v/F_m) \), indicative of chronic photoinhibition. However, there was no significant difference in \( F_v/F_m \) between tolerant and sensitive genotypes at both growth temperatures.

Figure 2 shows the effect of gradual cooling at moderate light intensity on the chlorophyll fluorescence characteristics of the leaves. The PSII operating efficiency \( (F_o/F_m') \) was clearly strongly inhibited at temperatures below 10 °C in leaves developed at 25 °C and at 15 °C. In leaves grown at 25 °C, this reduction was associated with a decrease in photochemical quenching factor \( (F_d/F_o') \) as well as in the efficiency of open reaction centers \( (F_o'/F_m') \). Sensitive and tolerant genotypes differed only slightly in their response to temperature when the leaves were developed at 25 °C. In contrast, tolerant and sensitive genotypes could be clearly separated on the basis of \( F_v/F_m' \) and \( F_o'/F_m' \) in leaves grown at 15 °C at temperatures below 8-10 °C. When compared with leaves grown at 25 °C, leaves grown at 15 °C were characterized by a lower efficiency of open reaction centers \( (F_o'/F_m') \) at all temperatures and by higher \( F_o'/F_m' \) values at temperature below 10 °C. The response of \( F_o'/F_m' \) was further investigated by exposing leaves to increasing illumination at 25, 15 and 6 °C (Figure 3). As in the previous experiment, measurements of leaves grown at 25 °C revealed little difference between genotypes under all measuring conditions. Increasing the light intensity resulted in a decrease in \( F_o'/F_m' \) which was accelerated at low temperature. For a given temperature, \( F_o'/F_m' \) was always lower in leaves grown at 15 °C than in leaves grown at 25 °C. Tolerant and sensitive genotypes, developed at 15 °C, could be separated best on the basis of \( F_o'/F_m' \) at light intensities below 400 µmol m\(^{-2}\) s\(^{-1}\) and at a measuring temperature of 6 °C. This promising result led us to determine whether \( F_o'/F_m' \) measured at low temperature could be used to select for the maintenance of photosynthetic capacity of plants developed at suboptimal temperature by breeding using the procedure described in Materials and methods. The result in Table 2 shows that this is the case for five out of the eight hybrids between selected \( F_3 \) families, the best being the dent H2 × H1 cross which showed a 31% increase of photosynthesis when compared with the original dent population when the plants were grown at suboptimal temperature. Two of the flint crosses also showed a significant increase in photosynthesis when compared with the original flint population when the plants were grown under optimal temperature. Three of the five crosses with improved photosynthetic capacity at suboptimal growth temperature also showed significantly higher chlorophyll contents than the original populations (Table 2).
Discussion

The three tolerant lines had higher rates of photosynthesis than the three sensitive lines when grown at suboptimal temperature, showing that there is much genetic variability within the Zea mays species as far as the adaptation of the photosynthetic apparatus to low growth temperature is concerned. In addition, the observation that the photosynthesis of leaves of two of the tolerant genotypes, grown at 15 °C, increased substantially when the temperature was switched from 15 °C to 25 °C (Figure 1 B, D) is of particular importance for plants growing under natural conditions. It suggests that tolerant genotypes may benefit immediately from a warmer temperature after a prolonged period of low temperatures. It is likely that they could better meet the increased demand for carbohydrates than the sensitive genotypes in order for growth to resume with increasing temperature. This hypothesis is in agreement with the observation that the two inbreds, Z7 and Z15, showed higher relative growth rates than the two sensitive inbreds, Penjalinan and CM109, when grown under cool conditions in the field (Verheul et al., 1996).

The poor photosynthetic performance of the sensitive lines grown at 15 °C was associated with a chlorophyll deficiency (Table 1) (Haldimann, 1998). The chloroplast development of the sensitive genotypes may be particularly sensitive to low temperature or,
alternatively, the pigments of sensitive genotypes might be destroyed because of a high sensitivity to oxidative stress induced by low temperature. The chilling tolerance of the genotypes was not correlated to the maximum efficiency of PSII photochemistry ($F_v/F_m$, Table 1) either in leaves grown at 25 °C nor in leaves grown at 15 °C, even though growth at suboptimal temperature led to a slight decrease of $F_v/F_m$. However, using $F_q'/F_m'$ to select for tolerance to severe chilling may be a good approach since there is a great genetic variation in tolerance to low temperature-induced photoinhibition in maize (Dolstra et al., 1994). The fact that the tolerant line Z7 had the lowest $F_q'/F_m'$ value of all the lines when grown at 15 °C, but a relatively high photosynthetic capacity, indicates that moderate photoinhibition hardly affects the photosynthetic rate at high light. Decreasing leaf temperature induced a decrease in photosynthetic electron transport activity, resulting in a clear difference between tolerant and sensitive genotypes in leaves developed at suboptimal temperature (Figure 2). Since all genotypes behaved similarly when the leaves were developed at optimal temperature, the genotypic difference must be related to adaptation mechanisms induced by suboptimal growth temperature. Constitutive differences probably also exist in maize. Genotypic variation in the response of $F_q'/F_o'$ to low temperature in leaves developed at 25 °C has been reported (Havaux, 1987). Interestingly, leaves of all genotypes grown at 15 °C maintained higher $F_q'/F_o'$ value than leaves grown at 25 °C in response to gradual cooling (Fig-

![Graph showing the effect of increasing light intensity on $F_q'/F_m'$ of maize leaves developed at 25 °C (A, C, E) and 15 °C (B, D, F) and measured at 6 (A, B), 15 (C, D) and 25 °C (E, F). Values are means (± SD) of five plants. See Figure 1 for legend.](image-url)
This suggests that protection mechanisms are present both in sensitive and in tolerant genotypes, allowing the leaves to prevent excessive reduction of PSII acceptors. It has been suggested that this feature is related to the high content of the xanthophyll zeaxanthin in maize leaves developed at suboptimal temperature (Haldimann et al., 1995). Many researchers (see, Demmig-Adams and Adams, 1996, for a review) reported that this pigment is related to excess energy dissipation as heat in the antennae. Thus, its presence in leaves developed at suboptimal temperature is probably also responsible for the reduced efficiency of excitation energy capture by open reaction PSII reaction centers (\(F_{v’/F_{m’}}\)) when compared with leaves grown at optimal temperature (Fryer et al., 1995). Furthermore, the difference in the behavior \(F_{q’/F_{m’}}\) in the genotypes (Figure 2) is largely due to a difference of \(F_{v’/F_{m’}}\), suggesting that xanthophyll cycle pigments might be related to the genotypic difference. The recent observation that, when leaves are grown at suboptimal temperature, sensitive genotypes accumulate more zeaxanthin than tolerant genotypes (Haldimann, 1998) supports this hypothesis. The greater capacity of the tolerant lines to maintain higher electron transport rates than sensitive lines at low temperature (indicated by \(F_{q’/F_{m’}}\)) does not necessarily mean that the quantum yield of carbon fixation of the tolerant lines was superior under these conditions, since the linear relation between \(F_{q’/F_{m’}}\) and the quantum yield of CO₂ fixation usually observed in maize (Genty et al., 1989; Massacci et al., 1995) can be deviated in leaves developed at low temperature in the field (Fryer et al., 1998). In the experiment presented in Figure 2, the lowest temperature was reached after around 2 h of gradual cooling, long enough for important changes within the photosynthetic apparatus to occur, such as the conversion of xanthophyll cycle pigments (Leipner et al., 1997). These changes may have significant effects on chlorophyll fluorescence quenching. For this reason, the response of \(F_{q’/F_{m’}}\) to temperature was further investigated by exposing leaves to light at 6, 15 or 25 °C (Figure 3). The results of the measurements made at 6 °C clearly confirm the genotypic difference observed in Figure 2. Furthermore, the response of \(F_{q’/F_{m’}}\) to increasing light intensity at 15 °C and 25 °C provides further information about the nature of this difference. The fact that leaves of tolerant and sensitive genotypes grown at 15 °C, behaved similarly at these two temperatures even under high illumination indicates that the cause of the genotypic difference visible at 6 °C is not expressed in response to excess energy per se, but is specific to low leaf temperature. Although the cause of the genotypic difference remains to be found, the clear difference between tolerant and sensitive genotypes, detected when leaves grown at 15 °C were exposed to low illumination (<200 µmol m⁻² s⁻¹) at 6 °C (Figure 3), suggests that \(F_{q’/F_{m’}}\) is a useful criterion for differentiating between maize plants with different chilling tolerance of photosynthetic capacity. The \(F_{q’/F_{m’}}\) of Triticum species of different origin has also been reported to differ substantially at low temperature (Rekika et al., 1997) and could be used to discriminate between wheat cultivars that differ in drought resistance (Flagella et al., 1996). Because \(F_{q’/F_{m’}}\) is directly related to the rate of electron transport, it might be a useful tool for selecting for resistance to different types of stress which inhibit photosynthesis. However, to the best of our knowledge, the use of \(F_{q’/F_{m’}}\) as a selection tool to improve the stress tolerance of photosynthesis through breeding has not yet been reported. Thus, it was interesting to determine whether its use in a

<table>
<thead>
<tr>
<th>Table 2: The net rate of CO₂ assimilation (A) and chlorophyll a+b content (Chl a+b) of leaves of hybrids between F₃ maize lines bred for chilling tolerance (H1 to H4) as well as of the original populations from which they are derived (O).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype Parameter</td>
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</tr>
<tr>
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<tr>
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<td>H₂ × H₁</td>
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<tr>
<td>H₄ × H₃</td>
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<tr>
<td>O</td>
</tr>
</tbody>
</table>

Measurements were performed under the growth conditions (25 °C or 15 °C, light intensity: 450 µmol m⁻² s⁻¹). Values are means (± SD) of 6 or 18 plants for the hybrids and the original populations, respectively. Stars indicate that, for a given growth temperature, the selected hybrid was significantly different from the original population in a t-test comparison. *: \(P < 0.05\); **: \(P < 0.01\); ***: \(P < 0.001\); NS: not significant at \(P < 0.05\).
breeding program would improve the chilling tolerance of photosynthesis in maize. The result, summarized in Table 2, shows that the use of $F_q/F_m'$ in breeding programs can substantially increase the photosynthetic capacity of maize plants developed at suboptimal temperature. The hybrids of $F_3$ families are already different to the original population, suggesting that only a limited number of genes are involved in the trait ($F_3$ are only $F_2$ inbreds because $F_1$ were hybrids). These traits might be dominant, or the selection method may have led to the independent selection for the same traits in the different families. The relatively small number of plants used at each selection step (maximum 40 for a selected ear, see Materials and methods) suggest that the method is very efficient, assuming that the genetic variation within the original population is large enough. Indeed, the variation in the photosynthesis of leaves developed at suboptimal temperature seems larger in the original flint population than in the dent population (see SD values in Table 2). This may explain why the selection program was more efficient with the flint population than with the dent population.

In several cases, selection also led to a significant increase in the leaf chlorophyll content of plants grown at suboptimal temperature compared with the original populations (Table 2). This is not very surprising in view of the pattern obtained with the model genotypes (Table 1). An increase in the tolerance of photosynthetic capacity may often occur with a concomitant increase in the ability to maintain high chlorophyll content at suboptimal growth temperature.
Chapter 4

Genetic analysis of chilling-tolerance of photosynthesis in maize seedlings under controlled conditions*

The genetic basis of chilling-tolerance was investigated by analyzing the quantitative trait loci (QTL) of an F_{2:3} population derived from a cross between two lines bred for contrasting chilling-tolerance using chlorophyll fluorescence as a selection tool. Chlorophyll fluorescence parameters, CO_{2} exchange rate, leaf greenness, shoot dry matter and shoot nitrogen content were determined in plants grown under controlled conditions at 25/22 °C or 15/13 °C (day/night). The analysis revealed the presence of 18 and 19 QTLs (LOD > 3.5) significantly involved in the variation of nine target traits in plants grown at 25/22 °C and 15/13 °C, respectively. Only four QTLs were clearly identified in both temperatures regimes for the same traits, demonstrating that the genetic control of the performance of the photosynthetic apparatus differed, depending on the temperature regime. A major QTL for the chilling-tolerance of photosynthesis was identified on chromosome 6. This QTL alone explained 37.4% of the phenotypic variance in the chronic photoinhibition at low temperature and was significantly involved in the expression of six other traits, including the rate of carbon fixation and shoot dry matter accumulation, indicating that the tolerance to photoinhibition is a key factor in the tolerance of maize to low growth temperature. An additional QTL on chromosomes 2 corresponded to a QTL identified previously in another population, suggesting some common genetic basis of the chilling-tolerance of photosynthesis in different maize germplasms.

Introduction

In temperate regions, the maize crop is often exposed to low temperature during early development, resulting in poor photosynthetic performance (Leipner et al., 1999a; Stirling et al., 1991) related to delayed plant development (Miedema, 1982). The cold-induced decrease in the photosynthesis of maize has been associated with the photo-damage of the PSII reaction centers (Haldimann et al., 1995; Ortiz-Lopez et al., 1990), alteration of the pigment composition of the leaves (Haldimann et al., 1995), increased dissipation of excess energy in the antennae of photosystem II (PSII) (Andrews et al., 1995), lower activity of enzymes in the carbon cycle (Kingston-Smith et al., 1997) and altered chloroplast development (Nie and Baker, 1991; Robertson et al., 1993). Various studies in which genotypes of different origin were compared suggested that there is considerable genetic variation in the chilling-tolerance of the photosynthesis of maize (Andrews et al., 1995; Haldimann, 1998) and that the rate of carbon exchange is a good indicator of early chilling tolerance (Lee et al., 2002a). This genetic variation was exploited to breed new lines with very different tolerance of the photosynthetic apparatus to low growth temperature (see Chapter 3 and Fracheboud et al., 1999).

Since about 10 years, molecular markers have enabled the identification of quantitative trait loci (QTL) which are involved in the expression of agronomically important traits of maize, such as yield components (Kraja and Dudley, 2000) or disease resistance (Moon et al., 1999). Furthermore, the genetic dissection of abiotic stress resistance, such as drought (see Ribaut et al., 2004 for a review) and to a lesser extent low nitrogen (Agrama et al., 1999; Hirel et al., 2001), is also well documented. In comparison, little is known on the genetic basis of chilling-tolerance which has been investigated only recently in maize (Fracheboud et al., 2002) and rice (Andaya and Mackill, 2003). A better knowledge of the genetic basis of chilling-tolerance in different maize germplasms would allow to identify the level of complexity for the different regulatory traits and would provide suitable information for the improvement of maize by marker assisted selection, especially if the QTLs identified express a large proportion of the phenotypic variance and are stable in different genetic backgrounds.

In the present study, a QTL analysis was carried out on an F_{2:3} population which was issued from the crossing of two maize lines bred for contrasting chilling-tolerance of photosynthesis. The aim was to assess a possible genetic link between growth and photosynthetic performance at low temperature. The

* based on the following publication:
results were compared with those for similar traits in another population of different origin (Fracheboud et al., 2002) in order to identify overlapping QTLs for the chilling-tolerance of photosynthesis between the two populations.

**Materials and methods**

**Plant material**

Parental lines with contrasting chilling-tolerance of photosynthesis were obtained by divergent selection from a Swiss dent breeding population using chlorophyll fluorescence as a selection tool (Chapter 3; Fracheboud et al., 1999). One chilling-tolerant S₅ line (ETH-DH7) and one chilling-sensitive S₅ line (ETH-DL3), the mother, were crossed; the hybrid was self-pollinated to produce an F₂ segregating population. A total of 254 F₂ plants were grown in the field and self-pollinated, yielding 226 F₃ families. Twenty plants of each F₃ were grown in the field and inter-crossed within families for seed multiplication and producing the F₂:₃ population used for the phenotypic evaluation of the QTL experiments.

**Growth conditions**

The plants were grown in a growth chamber (PGW36, Conviron, Winnipeg, Canada) in pots (10 × 10 × 10 cm) containing a commercial mixture of soil, peat and compost (Topf und Pikiererde 140, Ricoter, Aarberg, Switzerland). The plants were sown at intervals of 24 h in blocks of three plants of 22 families. Six to nine plants of each F₂:₃ family and six plants of each parental line and of their two reciprocal F₁ hybrids were analyzed at each growth temperature. The control plants grew for 13 days at 25/22 °C (day/night) and the genomic DNA was extracted according to the protocol of Hoisington et al. (1994). The linkage map was constructed using simple sequence repeats (SSRs) available in the public domain (Maize Genetics and Genomics Database) as genetic markers. The PCR amplification, the separation of amplified DNA on agarose gels and their detection were performed according to Hoisington et al. (1994) with the exception that Resophor and STG agarose (Eurobio, Les Ulis, France) was used instead of Metaphor and Saekem agarose. The genetic map was constructed using Mapmaker 3.0 (Lander et al., 1987) with the Haldane mapping function.

**QTL identification**

The QTLs were identified by composite interval mapping using QTL Cartographer 1.16 (Basten et al., 1994), model 6 (Basten et al., 2005), with a blocking
window size of 30 cM. The co-factors were selected by forward and backward regressions with the in and out thresholds at a P-value of 0.05. The presence of a QTL was considered to be significant in single trait analysis when the likelihood of odds (LOD) value was larger than 3.5. This value corresponds to a type I error rate (α) of 0.021 for a single trait analysis in an F2 population (three degrees of freedom), assuming that all chromosome arms segregate independently. In the joint analysis of plants grown at 15 and 25 °C, the presence of a QTL was considered to be significant when the LOD value was larger than 4.42 (also corresponding to a type I error rate of 0.021 for a two traits analysis with five degrees of freedom) and was considered to be stable across the two growth temperature regimes when the LOD value for the genotype × environment interaction was < 1.30 (corresponding to a type I error rate of 0.05). The raw segregation data and phenotypic observations are deposited at the Maize Genetics and Genomics Database.

**Results**

Table 1 gives the statistics of the traits of the plants grown at 25 and 15 °C, respectively. The traits of the two parental lines differed significantly when grown at 15 °C. Under these conditions, the superior chilling-tolerance of ETH-DH7 over ETH-DL3 is clearly demonstrated by the higher Fm'/Fm (indicative of better tolerance to chronic photoinhibition), greater operating quantum efficiency of PSII photochemistry Fv'/Fm', greater PSII maximum efficiency (Fv'/Fm'), higher proportion of open reaction centers, indicated by Fv'/Fv' (Joliot and Joliot, 1964), a higher carbon exchange rate (CER), greener leaves (SPAD) and greater shoot dry matter accumulation (shoot DW). In contrast, when grown at 25 °C, CER, Fm'/Fm' and the ground fluorescence of the dark-adapted leaves (F0) of the two lines did not differ significantly. Although the two lines showed significant differences for the other traits when grown at 25 °C, these differences were small, except for shoot DW, which was higher in the chilling-sensitive line ETH-DL3. No significant differences were found between the two F1 hybrids except for Fm'/Fm' at 15 °C, indicating that there was no maternal effect on most of the traits. The F2:3 families segregated significantly for all the target traits at both growing temperatures. A comparison of the traits of the parental lines and F2:3 families showed a clear transgressive distribution underlying the polygenic nature of the traits under study and a favorable genetic contribution of both parental lines. There were, however, two exceptions, Fm'/Fm' at 25 °C and Fm'/Fm' at 15 °C. As expected from the nature of the two parents and their different chilling-tolerance, the transgressive nature of the distribution was more marked at 25 °C than at 15 °C. The low heritability observed for CER and chlorophyll fluorescence parameters suggest that these traits were highly affected by the environment. The relationships between CER and the other photosynthesis-related traits are shown in Figure 1. The carbon exchange rate was best correlated with Fm'/Fm' and Fm'/Fm' at both growth temperatures. CER

<table>
<thead>
<tr>
<th>Trait</th>
<th>Growth temp (°C)</th>
<th>Parental lines</th>
<th>F1 hybrids</th>
<th>F2:3 population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ETH-DH7</td>
<td>ETH-DL3</td>
<td>ETH-DH7 × DL3</td>
<td>ETH-DL3 × DH7</td>
</tr>
<tr>
<td>Fv</td>
<td>25</td>
<td>0.180</td>
<td>0.179</td>
<td>NS</td>
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<td></td>
<td>15</td>
<td>0.386</td>
<td>0.510</td>
<td>***</td>
</tr>
<tr>
<td>Fv/Fm</td>
<td>25</td>
<td>0.775</td>
<td>0.761</td>
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<tr>
<td></td>
<td>15</td>
<td>0.718</td>
<td>0.490</td>
<td>***</td>
</tr>
<tr>
<td>Fm'/Fm</td>
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<tr>
<td></td>
<td>15</td>
<td>0.179</td>
<td>0.066</td>
<td>***</td>
</tr>
<tr>
<td>Fv'/Fv</td>
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<td>0.560</td>
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<td></td>
<td>15</td>
<td>0.257</td>
<td>0.107</td>
<td>***</td>
</tr>
<tr>
<td>Fm'/Fm'</td>
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<td>14.8</td>
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<td></td>
<td>15</td>
<td>3.5</td>
<td>9.0</td>
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</tr>
<tr>
<td>SPAD</td>
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<td>36.2</td>
<td>33.5</td>
<td>***</td>
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<td></td>
<td>15</td>
<td>23.1</td>
<td>12.3</td>
<td>***</td>
</tr>
<tr>
<td>Shoot DW</td>
<td>25</td>
<td>154</td>
<td>192</td>
<td>**</td>
</tr>
<tr>
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<td>15</td>
<td>5.3</td>
<td>5.6</td>
<td>*</td>
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Table 1: Summary statistics of the two parental lines, the two F1 hybrids and the F2:3 offsprings grown at 25 and 15 °C.
Figure 1: Relationship of the carbon exchange rate (CER) and the PSII operating efficiency ($F_{q'}/F_{m'}$), leaf greenness (SPAD), PSII maximum efficiency ($F_{v'}/F_{m'}$), photochemical quenching factor ($F_{q'}/F_{v'}$), ‘dark’ level of chlorophyll fluorescence ($F_o$) and maximum quantum efficiency of PSII primary photochemistry ($F_v/F_m$) in the third leaf of maize grown at 25 °C and 15 °C. White circles: F2:3 families grown at 15 °C; black circles: F2:3 families grown at 25 °C; gray triangles: tolerant parent ETH-DH7; gray squares: sensitive parent ETH-DL3. Values represent averages of six to nine replicates.
was also moderately correlated with SPAD and the quenching factor ($F_q/F_m'$) at both temperatures. In contrast, $F_o$ and $F_q/F_m$ showed a significant correlation with the CER only in plants grown at 15 °C, where the lines with a high CER were characterized by a high $F_q/F_m$ and a low $F_o$.

About 1200 SSRs with a known map location were tested on the two parental lines to identify polymorphic markers. To produce the maximum genome coverage 118 polymorphic markers were selected. The map obtained (Figure 2) was 1930 cM long with an average distance of 16.4 cM between two consecutive markers. As judged by the bin maps from the Maize Genetics and Genomics Database (http://www.maizegdb.org), the map covered the whole genome, except on chromosome 5 from bin 900 to bin 1180 where no polymorphic SSRs were identified. The relatively low polymorphism was attributed to the similar origin of both parental lines (Swiss Dent).

The QTLs identified in plants grown at 25 and 15 °C are shown in Tables 2 and 3. The QTL analysis of plants grown at 25 °C revealed the presence of 18 significant QTLs, identified at 11 loci, involved in the expression of eight of the nine analyzed traits (Table 2). The two major QTLs with high LOD scores and $R^2$ values were located on chromosomes 3 (94–112 cM) identified for $F_o$ and $F_q/F_m'$ and $F_o/F_m$. Beside these two loci, additional QTLs were identified on chromosomes 2 (9 and 124 cM), 3 (51 cM), 4 (141 cM), 6 (81–95 cM), 7 (67 cM) and 9 (188 cM). Except for $F_o$, positive values for additivity indicate that the favorable alleles were carried by the tolerant parental line ETH-DH7, while negative values indicate that the chilling-sensitive parental line ETH-DL3 carried the favorable allele, the situation being the opposite for $F_o$. Therefore, ETH-DH7 carried favorable alleles at four QTLs and ETH-DL3 carried favorable alleles at the other seven QTLs. The positive and negative signs of additivity at the different loci in about the same ratio indicate the genetic contribution of both parental lines and confirm the transgressive segregation observed at the phenotypic level. At 25 °C, the gene action at the loci varied from additive (e.g. chromosome 2, 124 cM for SPAD) to over-dominant (e.g. chromosome 6, 221 cM for $F_o$).

In plants grown at 15 °C, the variation in the traits appeared to be under different genetic control (Table 3). A total of 19 significant QTLs have been identified at nine loci for the nine target traits considered. A major locus was identified on chromosome 6 (209–221 cM), 2 (4–47 cM and 118–121 cM), 3 (51–53 cM), 4 (4 cM), 6 (120...
cM) and 8 (74 cM). In contrast to the situation at 25 °C where the contribution of the two parental lines to the expression of the different target traits was in a similar range, the chilling-tolerance of ETH-DH7, observed at the phenotypic level, is clearly confirmed at the genetic level. For the six photosynthetic traits and shoot DW, the signs of additivity indicated that the tolerant parent, ETH-DH7, carried favorable alleles at all loci except those one on chromosomes 2 (47 cM) for \( F_v/F_m \) and 4 (4 cM) for \( F_o/F_m \). The action of the genes at these loci varied from partially dominant to over-dominant, depending on the locus and trait considered.

The major locus of chromosome 6 was also detected in plants grown at 25 °C (Table 2), suggesting a constitutive expression for certain traits. Apart from this locus, only one QTL for the greenness of the leaf, detected on chromosome 2 at 124 cM, was identified significantly both under 25 and 15 °C, demonstrating that common genetic basis of both experimental conditions was reduced. This locus, which was involved in the regulation of CER at 15 °C, was also detected for \( F_o/F_m \) and SPAD at 15 °C. A third QTL, involved in the regulation of CER at low temperature, was located on chromosome 3 (51 cM). This locus was also identified for \( F_o/F_m \).

The analysis also identified a QTL with a very large R\(^2\) for \( F_o/F_m \) at 15 °C on chromosome 4 at 47 cM, but this locus was not involved in the variation of any other trait. The R\(^2\) value may be over-estimated because this trait did not follow a normal distribution (not shown).

The QTLs were also mapped in a joint analysis of both growth temperatures. This analysis allows computing an LOD score for genotype by environment interaction and therefore enables to determine if QTLs are stable across environments. Additionally, the joint analysis increases the power of QTL detection where LOD peaks below the detection threshold are present under both environments in single trait analysis. The result (Table 4) indicated that all QTLs identified for \( F_o \) and \( F_v/F_m \) at 25 °C (Table 2) were stable across temperature environments, but had modest effects on the phenotypic variance in plants grown at 15 °C. The analysis also revealed the presence of new QTLs stable across temperatures for \( F_o \) on chromosomes 1 (229 cM), 7 (77cM) and 10 (120 cM). The joint analysis also suggested that the QTL identified previously on chromosome 1 for \( F_o/F_m \) at 25 °C (Table 2) and SPAD at 15 °C (Table 3) is also involved in CER.

In order to compare the QTL data of the present study with those identified in the population Ac7643 × Ac7729 for similar traits under similar environments (Fracheboud et al., 2002), the QTLs in both populations were transposed to the IBM 2 neighbor consensus map available at the Maize Genomic and Genetic Database. The analysis revealed the presence of an interesting overlapping QTL on chromosome 2 identified for CER, \( F_v/F_m \) and SPAD at 15 °C in the present study (Table 3) and CER and \( F_o/F_m \) at 15 °C in Ac7643 × Ac7729. The overlap was confirmed by mapping the SSR \( bnlg1909 \) in Ac7643 × Ac7729 (Figure 3). The major QTL identified previously on chromosome 3 (Fracheboud et al., 2002), mapped close to the QTLs identified for CER at 25 °C (110 cM, Table 2) and CER at 15 °C (51 cM, Table 3).

### Table 2: Main characteristics of QTLs with an LOD score > 3.5 in plants grown at 25 °C.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Chr</th>
<th>Peak Interval</th>
<th>Nearest marker</th>
<th>LOD score</th>
<th>Add</th>
<th>Dom</th>
<th>R(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( F_o )</td>
<td>1</td>
<td>89 73-103</td>
<td>bnlg1886</td>
<td>4.2</td>
<td>0.002</td>
<td>0.004</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>81 59-110</td>
<td>bnlg1188</td>
<td>7.7</td>
<td>0.004</td>
<td>-0.004</td>
<td>17.7</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>221 199-239</td>
<td>bnlg1740</td>
<td>13.0</td>
<td>-0.003</td>
<td>-0.006</td>
<td>18.1</td>
</tr>
<tr>
<td>( F_v/F_m )</td>
<td>1</td>
<td>181 179-230</td>
<td>mmc0041</td>
<td>6.9</td>
<td>0.003</td>
<td>-0.004</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>94 79-120</td>
<td>mmc0022</td>
<td>6.5</td>
<td>-0.003</td>
<td>0.001</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>141 112-169</td>
<td>dupssr34</td>
<td>6.1</td>
<td>-0.003</td>
<td>-0.001</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>95 65-125</td>
<td>umc1887</td>
<td>8.3</td>
<td>-0.003</td>
<td>0.005</td>
<td>15.1</td>
</tr>
<tr>
<td>( F_v/F_m' )</td>
<td>3</td>
<td>102 74-140</td>
<td>mmc0022</td>
<td>6.9</td>
<td>-0.009</td>
<td>0.014</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>209 189-239</td>
<td>bnlg1740</td>
<td>5.6</td>
<td>0.012</td>
<td>0.015</td>
<td>18.9</td>
</tr>
<tr>
<td>( F_v/F_m' )</td>
<td>6</td>
<td>213 195-239</td>
<td>bnlg1740</td>
<td>9.9</td>
<td>0.013</td>
<td>0.022</td>
<td>32.8</td>
</tr>
<tr>
<td>( F_o/F_m' )</td>
<td>3</td>
<td>94 76-122</td>
<td>mmc0022</td>
<td>8.2</td>
<td>-0.010</td>
<td>0.002</td>
<td>15.0</td>
</tr>
<tr>
<td>CER</td>
<td>2</td>
<td>9 3-51</td>
<td>umc1823</td>
<td>3.7</td>
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<td>1.17</td>
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</tr>
<tr>
<td></td>
<td>3</td>
<td>110 98-146</td>
<td>bnlg1063</td>
<td>4.7</td>
<td>-0.63</td>
<td>0.10</td>
<td>10.8</td>
</tr>
<tr>
<td>SPAD</td>
<td>2</td>
<td>124 108-154</td>
<td>bnlg121</td>
<td>4.2</td>
<td>0.96</td>
<td>0.06</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>112 74-150</td>
<td>bnlg1063</td>
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<td>-1.37</td>
<td>0.54</td>
<td>15.5</td>
</tr>
<tr>
<td>shoot DW</td>
<td>3</td>
<td>51 42-67</td>
<td>bnlg1447</td>
<td>3.5</td>
<td>-6.4</td>
<td>16.2</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>67 64-85</td>
<td>bnlg2259</td>
<td>5.0</td>
<td>12.3</td>
<td>-0.2</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>188 134-188</td>
<td>umc1727</td>
<td>3.8</td>
<td>-13.6</td>
<td>-26.8</td>
<td>18.3</td>
</tr>
</tbody>
</table>

N(%) - - - - - -

\* Intervals were defined by the positions on the chromosome where the LOD score at the QTLs peaks decreased by half. Chr, chromosome number; Dist, QTL position in centimorgans; Add, additivity; Dom, dominance. R\(^2\), % of phenotypic variance explained by the QTL.
bnlg1063 in Ac7643 × Ac7729 did not demonstrate a clear overlap between the two populations.

**Discussion**

The two parental lines differed considerably in the rate of carbon exchange (CER) when grown at 15 °C but not at 25 °C (Table 1). This indicates that chlorophyll fluorescence was an efficient selection tool for different levels of chilling-tolerance of photosynthesis, thus confirming earlier observations (Fracheboud et al., 1999). Despite the similar CER of both lines when grown at 25 °C, their photosynthetic apparatus appeared to function differently at this temperature. The chilling-tolerant line ETH-DH7 maintained a high trapping efficiency of PSII ($F_{v'}/F_{m'}$) while ETH-DL3 maintained a higher proportion of open PSII centers,

**Table 3:** Main characteristics of QTLs with an LOD score > 3.5 in plants grown at 15 °C.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Chr</th>
<th>Peak</th>
<th>Interval</th>
<th>Nearest marker</th>
<th>LOD score</th>
<th>Add</th>
<th>Dom</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_o$</td>
<td>6</td>
<td>217</td>
<td>193-239</td>
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<td>-0.057</td>
<td>-0.049</td>
<td>37.2</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>74</td>
<td>55-93</td>
<td>mmc0181</td>
<td>5.3</td>
<td>0.026</td>
<td>-0.018</td>
<td>7.0</td>
</tr>
<tr>
<td>$F_o/F_m$</td>
<td>6</td>
<td>221</td>
<td>199-239</td>
<td>bnlg1740</td>
<td>21.6</td>
<td>0.033</td>
<td>0.031</td>
<td>37.4</td>
</tr>
<tr>
<td>$F_o/F_m'$</td>
<td>1</td>
<td>201</td>
<td>174-217</td>
<td>bnlg1502</td>
<td>3.5</td>
<td>0.010</td>
<td>0.023</td>
<td>6.7</td>
</tr>
<tr>
<td>$F_o/F_m''$</td>
<td>6</td>
<td>217</td>
<td>195-249</td>
<td>bnlg1740</td>
<td>5.1</td>
<td>0.016</td>
<td>0.002</td>
<td>10.3</td>
</tr>
<tr>
<td>$F_{v'}/F_m'$</td>
<td>2</td>
<td>118</td>
<td>100-144</td>
<td>bnlg1909</td>
<td>4.2</td>
<td>0.017</td>
<td>0.016</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
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<td>-0.002</td>
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<td>120</td>
<td>103-144</td>
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<td>0.033</td>
<td>5.8</td>
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<tr>
<td></td>
<td>6</td>
<td>219</td>
<td>197-239</td>
<td>bnlg1740</td>
<td>6.0</td>
<td>0.021</td>
<td>0.011</td>
<td>10.6</td>
</tr>
<tr>
<td>$F_{v'}/F_m''$</td>
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<td>35-61</td>
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<td>CER</td>
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<td>0.42</td>
<td>0.07</td>
<td>6.5</td>
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<tr>
<td></td>
<td>3</td>
<td>51</td>
<td>25-65</td>
<td>bnlg1447</td>
<td>3.6</td>
<td>0.35</td>
<td>0.27</td>
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</tr>
<tr>
<td></td>
<td>6</td>
<td>207</td>
<td>182-239</td>
<td>bnlg1740</td>
<td>3.7</td>
<td>0.46</td>
<td>-0.29</td>
<td>9.2</td>
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<tr>
<td>SPAD</td>
<td>1</td>
<td>176</td>
<td>146-207</td>
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<td>1.13</td>
<td>0.59</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>121</td>
<td>106-146</td>
<td>bnlg1909</td>
<td>6.4</td>
<td>1.43</td>
<td>1.12</td>
<td>10.0</td>
</tr>
<tr>
<td>shoot DW</td>
<td>6</td>
<td>219</td>
<td>194-239</td>
<td>bnlg1740</td>
<td>5.9</td>
<td>9.6</td>
<td>12.9</td>
<td>14.0</td>
</tr>
<tr>
<td>N(%)</td>
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<td>4</td>
<td>0-57</td>
<td>bnlg1017</td>
<td>3.5</td>
<td>-0.132</td>
<td>-0.237</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>201</td>
<td>180-224</td>
<td>umc1859</td>
<td>4.1</td>
<td>-0.199</td>
<td>-0.012</td>
<td>10.8</td>
</tr>
</tbody>
</table>

See Table 2 for legends.

**Figure 3:** Transposition of QTLs identified on chromosome 2 for CER at 15 °C in two maize populations mapped for chilling-tolerance on the IBM 2 neighbor map. Blue segments represent the QTL interval as described in Table 3 for the ETH-DL3 × ETH-DH7 population and the QTL interval recalculated from Fracheboud et al. (2002) for the Ac7643 × Ac7729 population.
Table 4: Main characteristics of stable QTLs across temperatures identified with an LOD score > 4.42 and an LOD for genotype × environment interaction (G×E) < 1.30 in the joint analysis of plants grown at 15 and 25 °C.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Chr</th>
<th>Peak</th>
<th>Interval</th>
<th>Nearest marker</th>
<th>LOD score</th>
<th>LOD (G×E)</th>
<th>Add</th>
<th>Dom</th>
<th>25 °C</th>
<th>15 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CER</td>
<td>1</td>
<td>181</td>
<td>165-207</td>
<td>dupssr12</td>
<td>8.3</td>
<td>0.90</td>
<td>0.032</td>
<td>-0.0019</td>
<td>10.2</td>
<td>0.1</td>
</tr>
<tr>
<td>SPAD</td>
<td>1</td>
<td>167</td>
<td>147-207</td>
<td>umc1128</td>
<td>5.0</td>
<td>0.67</td>
<td>0.86</td>
<td>0.07</td>
<td>3.7</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>121</td>
<td>104-130</td>
<td>bnlg1909</td>
<td>6.4</td>
<td>0.94</td>
<td>0.92</td>
<td>0.25</td>
<td>5.5</td>
<td>10.0</td>
</tr>
</tbody>
</table>

No QTLs were identified for \(F_q/F_m', F_v/F_m', F_o/F_m', \) shoot DW and N(%). Add, mean additivity; Dom, mean dominance; \(R^2\), % of phenotypic variance explained by the QTL.

indicated by \(F_q'/F_m'\). As result, the product of the two components, \(F_v/F_m\), did not differ significantly and resulted in similar rates of carbon fixation. The QTL analysis confirms this observation: of the two QTLs for \(F_q/F_m'\) one was also found for \(F_q/F_m\), while the other was detected for \(F_q'/F_m'\) (Table 2).

The correlations between CER and the other photosynthetic traits (Figure 1) strongly confirm previous observations of another population grown under similar conditions (Fracheboud et al., 2002). As in this previous study, the CER was highly correlated with \(F_q'/F_m'\) at both growth temperatures, and was positively correlated with \(F_v/F_m\) and negatively correlated with \(F_o\) in plants grown at low temperature (Figure 1). A decrease in the \(F_v/F_m\), associated with an increase in \(F_o\), is an indicator of photoinhibition due to damage of the PSII centers (Krause, 1988).

The relationship between low CER and \(F_v/F_m\) and high \(F_o\) may reflect a disturbance in or damage of the structure of the thylakoid membrane in the most susceptible lines, resulting in a poor maximum quantum efficiency of PSII, \(F_v/F_m\) (Fracheboud et al., 2002). Such structural damage has been observed in maize grown at low temperature by means of immunocytochemistry (Robertson et al., 1993). This suggests that the mechanisms of tolerance are similar in both populations, although the germplasms are quite different.

The joint QTL analysis (Table 4) revealed that, except the two QTLs for SPAD and the QTL for \(F_o\) on chromosome 8, none of the QTLs identified at 15 °C were stable across the two growth temperatures, indicating that most QTLs identified in plants grown at 15 °C (Table 3) were specific to low growth temperature. In contrast to chloroplast functioning, chlorophyll accumulation was found to be relatively stable across temperatures; three of the four QTLs, which were identified for this trait in the single trait analysis (Tables 2 and 3), were also present in the joint analysis. This is consistent with the higher heritability of SPAD compared to the chlorophyll fluorescence parameters (Table 1).

Since photoinhibition and photosynthetic activity are functionally related, the involvement of the QTL on chromosome 6 in \(F_v/F_m\), \(F_q/F_m\), \(F_v/F_m'\) and CER in plants grown at 15 °C indicates a strong pleiotropic effect. However this QTL was probably not involved in chlorophyll accumulation since there was no significant increase of LOD at this locus for SPAD at both temperatures. One hypothesis is that a favorable allele at this QTL contributes to the development of functional chloroplasts at low growth temperature or protects them because of its involvement in \(F_o\) and \(F_v/F_m\) (Table 3). This would result in a greater efficiency of trapping efficiency of PSII (\(F_v'/F_m'\)) and finally to a higher rate of CO₂ fixation. Alternatively, the gene at this QTL may be directly involved in carbon fixation or indirectly affected by feedback inhibition of carbon fixation. In this case, the presence of a negative allele that limits photosynthesis would result in an excess of energy on the PSII reaction centers leading to photoinhibition. Whatever the primary target of the gene operating at this locus, its impact on photosynthesis was sufficient to influence the growth rate of the plants, as shown by its involvement in the shoot dry matter at 15 °C. Thus this QTL was involved in the vigor of the seedlings at low growth temperature. This QTL was also identified for \(F_o\) and \(F_q'/F_m'\) in plants grown at 25 °C (Table 2), but the presence of a positive or a negative allele had no impact on \(F_v/F_m\) and CER or shoot DW. While shoot DW was controlled by the main locus involved in photosynthesis in plants grown at 15 °C, growth was under different genetic control in plants grown at 25 °C. This clearly shows that photosynthesis can limit the growth of plants at low temperature. The
main QTL on chromosome 6, responsible for chilling-tolerance, was also detected for the relative nitrogen content of the shoot at 15 °C, but with a negative sign of additivity (Table 3). This means that the allele carried by the tolerant line ETH-DH7 contributes to an increase in growth and photosynthesis and a decrease in the nitrogen content of the shoot. Therefore, chilling sensitivity of this population is not due to nitrogen deficiency, as it was suggested for soybean (Purcell et al., 1987). Looking at the Maize Genetics and Genomics Database, an interesting candidate gene for this major QTL is identified: *agp2*, coding for the leaf ADP glucose pyrophosphorylase small sub-unit. ADP glucose pyrophosphorylase is a key enzyme in the regulation of the starch turn-over within the chloroplast (Baroja-Fernández et al., 2001). Leaf starch represents a transient store, whose synthesis helps to avoid P limitation of photosynthesis. This regulatory starch turn-over cycle can also be seen as an over-flow pathway when sucrose export is limited. In this context it is interesting that *Arabidopsis* mutants with lower ADP glucose pyrophosphorylase activity are characterized by a low CER, low \( F_o/F_m \) and slow leaf growth (Sun et al., 1999). Similar results were found in potato leaves (Hattenbach et al., 1997); antisense repression of the small sub-unit of ADP glucose pyrophosphorylase resulted in a strong decrease of the photosynthetic capacity. An effect of reduced ADP glucose pyrophosphorylase activity on chlorophyll content was observed only in the transformant with an almost complete suppression of ADP glucose pyrophosphorylase activity while, in the transformant with half the activity of the wild type, the chlorophyll content was not affected. Since ADP glucose pyrophosphorylase will probably not be totally inhibited in our maize plants carrying the unfavorable allele, it would explain why the QTL at the end of chromosome 6 was associated with photosynthetic efficiency, but not with SPAD.

The second most important locus involved in the tolerance of the photosynthetic apparatus to low growth temperature, which was identified for \( F_o/F_m \), CER and SPAD, was located on chromosome 2 (118-121 cM). Although it was not significant, a peak was also present at this locus for \( F_o/F_m \). It was also involved in the greenness of the leaves (SPAD) in plants grown at 25 °C but without effect on the CER. Its low LOD (G × E) value in the joint analysis (Table 4) confirms that the gene at this locus might be constitutively involved in the accumulation of chlorophyll in this population.

There is always a chance that QTLs co-localize by chance across populations, however, considering the reduced number of QTLs identified per trait and the limited amount of total genome they cover (about 5% for each population in our studies) this probability remains low. Therefore, the ca. 20 cM overlap of the QTLs identified for CER on chromosome 2 in the two populations (Figure 3) indicates that the same gene(s) may be involved in the chilling-tolerance of both populations although they are from very different origins. A possible candidate gene at this locus is *ssu2* (ribulose bisphosphate carboxylase small sub-unit 2), located 15 cM below *bnl1909* on the map BNL 2002 in the Maize Genetics and Genomics Database. This hypothesis confirms the observation that this QTL is involved in the trapping efficiency of PSII in the light (\( F_o/F_m \)) but not in the maximum PSII efficiency measured in the dark (\( F_o/F_m \), Table 3). Changes in the activity of ribulose bisphosphate carboxylase can affect the photosynthetic activity of C4 species, as demonstrated for *Flaveria bidentis* transformed with an antisense construct of the small sub-unit of Rubisco (Kubien et al., 2003). Even the chlorophyll content decreased in the transformed plants indicating that the pigment composition can be adjusted to the capacity of the dark reactions. An other example is the *Bundle sheath defective2* (*Bds2*) gene of maize; its protein is required for the post-translational regulation of the Rubisco large sub-unit. The mutation of *Bds2* prevents the accumulation of Rubisco and, indirectly, affects the chlorophyll content (Brutnell et al., 1999).

A third QTL involved in CER of plants grown at 15 °C was located on chromosome 3 at 51 cM, not very far from the main QTL of CER in plants grown at 25 °C (110 cM). The two probably represent different loci, because their signs of additivity differ (Tables 3 and 4) and because the sign of additivity for plants grown at 15 °C changes at around 80 cM (not shown). The observation that the positions of these is close to the position of a main QTL for chilling-tolerance of photosynthesis identified earlier (Fracheboud et al., 2002) indicates that this region near the centromer of chromosome 3 contains important genes for the temperature adaptation of the photosynthetic apparatus of maize.

In both studies, a major QTL was identified, which was common to several photosynthetic traits. The fact that it was the main QTL for \( F_o/F_m \) suggests that tolerance to photoinhibition is a major factor in the tolerance of the photosynthetic apparatus of maize to low growth temperature. In the present work the variation in \( F_o \) and \( F_o/F_m \) was related mainly to a QTL on chromosome 6 (detected at 217 cM for \( F_o \) and 221 cM for \( F_o/F_m \), Table 3), while it was associated with a QTL on chromosome 3 in the previous study, suggesting that at least two main independent loci can control tolerance to chilling-induced chronic photoinhibition in maize. Further studies of other germplasm may lead to the identification of additional useful loci for the improvement of the chilling-tolerance of maize by marker-assisted selection. The different genetic control observed between the two growth temperatures in this study strongly suggest that an
improvement in chilling-tolerance can be achieved without a negative impact on the performance of the plants at optimal temperature. It also suggests that selection must occur at low temperature since there might be no spill-over between performances at optimal and at low temperature.

The identification of major QTLs with a high $R^2$ in two different germplasms suggests that the improvement of chilling-tolerance by marker-assisted selection is feasible. However, since these QTLs were identified in growth chambers, their usefulness should first be evaluated under field conditions.
Chapter 5

Genetic analysis of chilling tolerance in maize seedlings grown under field conditions*

The effect of low growth temperature on morpho-physiological traits of maize was investigated by the means of a QTL analysis in a segregating F2:3 population grown under field conditions in Switzerland. Chlorophyll fluorescence parameters, leaf greenness, leaf area, shoot dry weight and shoot nitrogen content were investigated at the seedling stage for two years. Maize was sown on two dates in each year; thus, plants sown early were exposed to low temperature, whereas those sown later developed under more favorable conditions. The main QTLs involved in the functioning of the photosynthetic apparatus at low temperature were stable across the chilly environments and were also identified under controlled conditions at suboptimal temperature in a previous study. Based on the QTL analysis, relationships between chlorophyll fluorescence parameters and leaf greenness were moderate. This indicates that the extent and functioning of the photosynthetic machinery may be under different genetic control. The functioning of the photosynthetic apparatus in plants developed at low temperature in the field did not noticeably affect biomass accumulation; since there were no co-locations between QTLs for leaf area and shoot dry weight, biomass accumulation did not seem to be carbon-limited at the seedling stage under chilly conditions in the field.

Introduction

During the latter part of the last century, the cultivation of maize (Zea mays L.), which originated in the subtropics, has been extended to higher latitudes. Adaptation to growth conditions, as in northern Europe, has been partially successful due to breeding for early maturing maize plants and, therefore, reaching a compromise between the risk of yield loss and an acceptable level of yield (Stamp, 1986). Nevertheless, the chilling-sensitive nature of maize makes early plant establishment in spring difficult under cool environmental conditions. Among the various effects of low temperature on the physiology of maize, that on the photosynthetic apparatus is considered to be especially important (Baker et al., 1994). In particular, the combination of high light intensity and low temperature, as occurs frequently during the early growing season in temperate regions, can cause photoinhibition of photosynthesis (Farage and Long, 1987). Leaves of maize, which develop under such conditions, are characterized by a lower photosynthetic capacity, lower quantum efficiency of CO2-fixation ($\Phi_{CO2}$) and lower photosystem II operating efficiency ($F_{q'}/F_{m'}$) than leaves which develop under more favorable conditions (Fryer et al., 1998; Leipner et al., 1999a; Nie et al., 1992). One reason for the lower photosynthetic performance might be the perturbation of chloroplast development, specifically, the limited ability of maize leaves to develop a functional photosynthetic apparatus at low temperature (Nie and Baker, 1991). Besides this, the susceptibility of the enzymes involved in the C4 cycle, especially that of pyruvate orthophosphate dikinase, are discussed as being the cause of the chilling sensitivity of the photosynthetic machinery of C4 plants and in particular of maize (for a review see Long, 1983). Moreover, ribulose bisphosphate carboxylase (Rubisco) activity is known to be reduced in maize seedlings at low temperature (Kingston-Smith et al., 1997). Its activity has been found to be the limiting factor for photosynthesis at suboptimal temperature in another C4 plant, Muhlenbergia glomerata (Kubien and Sage, 2004). Recently, the necessary intercellular partitioning of the antioxidative defenses between the mesophyll and bundle sheath and probably also a potential disruption of the circadian regulation of certain photosynthetic enzymes are claimed to cause the chilling sensitivity of maize (Foyer et al., 2002). Furthermore, the transport rate of assimilates between the mesophyll and the bundle sheath, as well as phloem loading, are considered to be affected by low temperature (Sowinski et al., 1998; Sowinski et al., 2003). Whatever is the primary cause of the chilling-induced reduction of photosynthesis, its effect on seedling growth has only been studied by the comparison of a few genotypes with contrasting chilling tolerance.

* based on the following publication:
(Verheul, 1992; Verheul et al., 1995), which does not yet allow a final conclusion to be drawn concerning the importance of photosynthesis for growth under low temperature conditions.

For some years, the use of molecular markers has enabled the identification of quantitative trait loci (QTLs) involved in the expression of important agronomic traits (e.g. yield components) (Kraja and Dudley, 2000) or disease resistance (Moon et al., 1999). Although the genetic dissection of drought tolerance in maize has been studied extensively (for a review see Ribaut et al., 2004), less is known about the genetic basis of chilling tolerance: research under controlled conditions has only recently been undertaken in this laboratory (Fracheboud et al., 2002; Fracheboud et al., 2004). Not only is QTL analysis important in breeding programs, it is also a powerful tool for studying the relationships between complex physiological traits (for a review see Prioul et al., 1997). Although complex processes like biomass accumulation are controlled by a large number of genetic factors, only a few of these processes are supposed to be limiting factors. Moreover, the importance of genetic factors for a certain trait might be considerably affected by environmental conditions, making the genetic dissection of physiological traits by means of QTL analysis a promising approach for stress physiologists.

Studies of chilling stress conducted under controlled conditions, however, often poorly reflect natural environmental conditions which are usually characterized by considerable fluctuations in temperature and light intensity. Hence, it is not surprising that growth of maize at an early stage of development under field conditions often shows little correlation with maize grown under controlled conditions (Revilla et al., 1998). Therefore, the aim of the present study was to conduct a QTL analysis in order to understand the genetic and physiological mechanism(s) of chilling tolerance of maize seedlings under field conditions. To do this, a segregating population, which was studied previously under controlled conditions (see Chapter 4 and Fracheboud et al., 2004), was sown on two different dates under temperate climatic conditions in Switzerland. Thus, plants sown early were exposed to chilling conditions, whereas plants sown later developed under conditions more favorable for growth. The QTL analysis of morpho-physiological traits and the identification of potential candidate genes may help to understand the relationship between photosynthesis and growth in different environments as well as the genetic background of chilling tolerance in maize.

Materials and methods

Plant material

Maize (Zea mays L.) lines with contrasting chilling-tolerance of photosynthesis were obtained by divergent selection from a Swiss dent maize breeding population using chlorophyll fluorescence as the selection tool (Chapter 3; Fracheboud et al., 1999). Two lines in the S₅ generation, namely ETH-DH7 (chilling-tolerant) and ETH-DL3 (chilling-sensitive) were used as parents to produce a segregating F₂ population. From 254 F₂ plants a genetic linkage map was constructed using simple sequence repeat markers (Chapter 4; Fracheboud et al., 2004). The F₂ plants were grown in the field and selfed, yielding 226 successful F₃ families. Of each F₃ family, 20 plants were grown in the field and intercrossed within families to produce the F₂₃ population used in the present QTL experiments.

Field experiments

In 2002, the F₂₃ families were sown on two dates: 26 April (early sowing) and 24 May (late sowing). In 2003, sowing was done on 14 April (early) and 15 May (late). The experimental unit was a single row plot with 50 plants, 5 m long and 0.75 m between the rows. All the experiments were over-planted by machine and later thinned to the final plant number. Trials of 226 F₂₃ lines and the two parental lines were conducted using an alpha (0,1) lattice design with 23 blocks per replication (Patterson and Williams, 1976) and two replications for each sowing date. Each replication was bordered by two rows of a mixture of the F₂₃ families. In each experimental unit, the first two plants were considered to be border plants and were not used for measurement. Field experiments were conducted at the experimental station of the Institute of Plant Sciences of the ETH in Eschikon near Zurich (47°26’ N, 8°40’ E, 550 m above sea level). The soil was an Eutric Cambisol (FAO classification) with a clay loam (CL) texture and a low content of organic matter (3%) (Richner et al., 1996). Air temperature (thermistor YSI 400, Yellow Spring Instruments, Yellow Spring, OH, USA) and global radiation (BF2, Delta-T Devices, Cambridge, UK) were recorded at 15 min intervals, 2 m above the soil surface close to the experimental site.

Photosynthesis and chlorophyll fluorescence

To obtain light response curves from the parental lines, carbon exchange and chlorophyll a fluorescence were monitored with a portable photosynthesis system (LI-6400) equipped with a LI-6400-40 pulse-amplitude modulation fluorometer (LI-COR, Lincoln, NE, USA). Light response curves were conducted at plants from the late sown set at the second leaf of seedlings in the 2nd leaf stage (11–12 June 2002) as well as during flowering at the second leaf above the
ear (2–3 August 2002). The photosystem II operating efficiency \( (F_a'/F_m') \) was calculated according to Genty et al. (1989). Other chlorophyll a fluorescence parameters were calculated using the ‘lake’ model according to Kramer et al. (2004b). The fraction of open PSII reaction centers was estimated as \( q_L = ((F_m' - F_o')/F_m') \), the quantum efficiency of open PSII reaction centers as \( \Phi_L = ((F_m' - F_o')F_o') / (F_m' \times F_o') \), the quantum efficiency of dissipation by down-regulation as \( \Phi_{DQ} = 1 - F_a'/F_m' - 1/(F_m'/F_m' + q_L(F_m'/F_o' - 1)) \) and the quantum efficiency of other non-photothermal losses (non-light-induced, basal or dark, quenching processes) as \( \Phi_{NO} = 1 - F_a'/F_m' - \Phi_{DQ} \).

For QTL analysis in 2002, the PSII operating efficiency \( (F_a'/F_m') \) of the F2:3 population were recorded with the Li-6400. The light intensity was set at 400 \( \mu \text{mol m}^{-2} \text{s}^{-1} \); the temperature of the sample chamber was 18 °C for plants sown early and 30 °C for plants sown late. These temperatures reflected the temperature in the field at the time of the measurements. Humidity and CO2 concentration were the same as the ambient conditions. The chlorophyll a fluorescence was measured in the middle section of the third leaf after about 2 min adaptation to chamber conditions.

In 2003, the chlorophyll a fluorescence was recorded with a pulse amplitude modulation fluorometer (PAM-2000, Walz, Effeltrich, Germany). The PSII operating efficiency \( (F_a'/F_m') \) was measured from leaf discs; the discs had been punched from the middle of the third leaf and incubated, while floating on water, in a growth chamber for at least 30 min. The temperature of the growth chamber was 18 °C (early sowing date) and 25 °C (late sowing date). The light intensity was 400 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). The \( F_a'/F_m' \) of six leaf discs of each experimental unit was measured. Furthermore, the maximum quantum efficiency of PSI primary photochemistry \( (F_o'/F_m) \) of the third leaf was determined in vivo on the F2:3 population in the field. \( F_a'/F_m \) was measured by applying a 1 s saturation flash (\( > 8000 \mu \text{mol m}^{-2} \text{s}^{-1} \)). The \( F_a'/F_m \) measurements were conducted during the night from about 1 h after sunset until about 1 h before sunrise. In the plants sown early, \( F_a'/F_m \) was measured from the evening of 16 May 2003 until the early morning of 18 May 2003. Measurements of the late-sown set were done from 6 June 2003 (evening) until 9 June 2003 (early morning). The \( F_a'/F_m \) of 10 plants was determined for each experimental unit.

**Quantitative trait loci analysis**

Means of the phenotypic traits of the F2:3 were checked for normality of distribution as described by Shapiro and Wilk (1965) using the SAS PROC UNIVARIATE (SAS 8.2, SAS Institute, 1999–2001, Cary, NC, USA). The adjusted mean for QTL analysis was calculated by the program Alphagen version 2.3 (Scottish Agricultural Statistics Service, Edinburgh). All the QTL analyses for the individual environments were performed using QTL Cartographer version 2.3b (Basten et al., 1994). The method of composite interval mapping (CIM), model 6 of the Zmapqtl program module, was deployed for mapping the QTLs and estimating their effects (Basten et al., 2005). The genome was scanned at 2 cM intervals and the window size was set at 30 cM. Cofactors were chosen using the forward–backward method of step-wise regression at \( p(F_m) = p(F_{out}) = 0.05 \). A joint analysis of the phenotypic data for early and late sowing in both years, respectively, made it possible to evaluate the QTL-by-environment (Q × E) interaction (Jiang and Zeng, 1995). The presence of a QTL was declared significant when the likelihood of odds (LOD) value was higher than 3.50 for a single trait analysis and higher than 4.43 for a joint analysis. These values correspond to a Type-I error rate (\( \alpha \) level) of 0.021, assuming that all the chromosome arms segregate independently. An LOD threshold of 1.3 for a significant Q × E interaction was based on the Type-I error rate of a single locus for an F2 with...
QTL analysis in field grown maize seedlings

Additive effects of the detected QTLs were also estimated by the Zmapqtl procedure of QTL Cartographer using hypothesis 31 (Basten et al., 2005). The R² value (coefficient of determination) from this analysis indicated the percentage of phenotypic variance explained by marker genotypes at the locus.

Results

Environmental conditions

Figure 1 shows the daily mean temperature and daily global radiation throughout the experiments. In 2002, the average temperatures of 14 d before the harvest of the seedlings were 13.3 °C (early sowing) and 19.0 °C (late sowing). In 2003, the average temperatures were 13.9 °C (early sowing) and 22.0 °C (late sowing). Global radiation fluctuated throughout the seedling development, especially in the early-sown sets. The average global radiation of 14 d before the harvest of the seedlings were 18.6/18.4 MJ m⁻² d⁻¹ (2002/2003) for the early sowing and 24.2/27.3 MJ m⁻² s⁻¹ (2002/2003) for the late sowing.

Characterization of the parental lines

In the first year of the experiment (2002), the photosynthetic apparatus of the parental lines was studied by light response curves. The light response of photosynthesis showed that the photosynthetic capacity and efficiency of the chilling-sensitive genotype, ETH-DL3, were lower than those of the chilling-tolerant genotype, ETH-DH7, in young seedlings, which developed at low temperature (Figure 2). Prior to measurement of the light response curves at the 2nd leaf stage, the average temperature of the 7 d before the measurements was 13.9 °C. The lower PSII operating efficiency ($F'_{q}/F_m$) in the sensitive compared with the tolerant genotype was mainly due to the lower quantum efficiency of open PSII reaction centers ($\Phi q_L$) and, to a lesser extent, to a decrease in the fraction of open PSII reaction centers ($q_L$). The difference in the efficiency of open PSII reaction centers seemed to be caused for the most part by non-light-induced quenching processes, as indicated by the higher value for $\Phi_{q_{NO}}$ in the sensitive line, while the light response of the quantum efficiency for dissipation by down-regulation ($\Phi_{NPQ}$) revealed only a few differences between the two genotypes. The comparison with leaves developed at favorable temperature (2nd leaf above the ear during flowering) showed that differences between the two genotypes disappeared under optimal temperature conditions. Moreover, seedlings of the chilling-tolerant genotype, ETH-DH7, were characterized by a higher maximum quantum efficiency of PSII primary photochemistry ($F_v/F_m$) and higher chlorophyll content; the latter was estimated from measurements of the leaf greenness (SPAD), especially of the early-sown sets (Table 1). The shoot dry weight and area of the third leaf was higher for the tolerant than for the sensitive genotype when plants were sown early. The opposite was observed when plants were sown late (Table 1).

Quantitative trait loci analysis

In the first experimental year, traits which characterize the photosynthetic apparatus and which can be measured in relatively short time, namely SPAD and $F'_{q}/F_m$, were determined in the segregating population. Interim conclusions, which were drawn after the first year and according to the results obtained under controlled conditions (Chapter 4; Fracheboud et al., 2004), revealed additional traits of great interest for examination in the second experimental year. In the
For leaf greenness (SPAD) measured on the third leaf, six QTLs were detected from the seedlings sown early (Table 2). These QTLs were located on chromosomes 1 (181 cM), 2 (125 cM), 3 (102 cM), 4 (20 and 138 cM) and 10 (78 cM). An increase in leaf greenness was due to the alleles of the chilling-tolerant parent at most of these loci, except for the QTL at chromosome 3 and that at the beginning of chromosome 4. At the latter two loci, the greener leaves were due to the allelic contribution of the chilling-sensitive parent, ETH-DL3. The QTL for leaf greenness on chromosome 2 was not present in plants sown late. The joint analysis revealed that this QTL was stable across cold environments, indicated by the low Q × E interaction for the early sowing dates (Table 2). In plants sown late, four QTLs for leaf greenness were revealed. Three were also found in the plants sown early. Only the QTL at chromosome 9 (28 cM) was specific for the late-sown plants.
QTLs for $F_q/F_m$ were found mostly in plants sown early (Table 2). The most prominent QTL for $F_q/F_m$ was located on chromosome 6 at 225 cM; it was found in both years and explained 7.6% of the phenotypic variance of $F_q/F_m$ in 2002 and 20.4% in 2003. The increase in this trait was due to the allelic contribution of the chilling-tolerant parent. There were further QTLs for $F_q/F_m$ on chromosomes 2, 4, 8 and 9 detected in the early sown sets (Table 2). One of these QTLs, namely the one at chromosome 8, was also found in the late-sown set. In both sets it had a very low Q × E interaction indicating a high stability. The QTL at chromosome 9 seemed to be specific to the early-sown plants in 2002; here, it explained a large percentage of the phenotypic variance and was characterized by a high additivity.

Measurements of the maximum quantum efficiency of PSII primary photochemistry ($F_v/F_m$) were conducted in 2003. Two QTLs for $F_v/F_m$ were common in both the early and the late-sown sets; they were located on chromosome 2 (138 cM) and chromosome 6 (225, 219 cM) (Table 3). These two QTLs explained a high proportion of the phenotypic variance in the early and late-sown sets. However, their additivity was high only in the early-sown plants, while it was rather low in the late-sown set. Two additional QTLs were found for the plants of the early sowing date; they were located at the beginning of the chromosomes 1 and 4. The additivity of these QTLs was negative, showing that the chilling-sensitive genotype carried the favorable allele for this trait. A decrease in $F_v/F_m$ can be caused by an increase in $F_a$ or a decrease in $F_m$. The QTL analysis revealed that the major QTL for $F_v/F_m$ was located on chromosome 6 but not for $F_a$. Since the additivity at this locus was positive for $F_v/F_m$ and negative for $F_m$, the decrease in $F_v/F_m$ seems to be due to an increase in $F_a$. By contrast, the QTL for $F_v/F_m$ at chromosome 4 was found also for $F_m$, but not for $F_a$. Since the additivity was negative for $F_v/F_m$ as well as for $F_m$, the decrease in $F_v/F_m$ seemed to be caused by a decrease in $F_m$ at this locus.

The QTL analysis revealed four QTLs for the area of the third leaf of the early-sown $F_{2:3}$ families and

Table 2: Main characteristics of Q TLs for leaf greenness (SPAD) and PSII operating efficiency ($F_q/F_m$) of maize seedlings sown early or late with an LOD score above a threshold of 4.43 for the joint analysis.

<table>
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<th>LOD score</th>
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<th>Add</th>
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Chr, chromosome number; cM, position of the peak of the QTL in centimorgans; Range, range of the QTL above the threshold LOD score; Joint, LOD score in the joint analysis of year 2002 and 2003; Q × E, LOD score value for QTL–environment interaction in the joint analysis of year 2002 and 2003; $R^2$, percentage of the phenotypic variance explained by genotype class at LOD peak; Add, Additivity (positive additivity = high values of the trait were inherited from the tolerant parent; negative additivity = high values of the trait were inherited from the sensitive parent).
Table 3: Main characteristics of QTLs with an LOD score >3.5 for photosynthetic maximum quantum efficiency of PSII primary photochemistry ($F_{v}/F_{m}$), minimum fluorescence ($F_s$), and maximum fluorescence ($F_m$) of the field experiment in 2003.

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Chr, chromosome number; cM, position of the peak of the QTL in centimorgans; Range, range of the QTL above the threshold LOD score; $R^2$, percentage of the phenotypic variance explained by genotype class at LOD peak; Add, Additivity (positive additivity = high values of the trait were inherited from the tolerant parent; negative additivity = high values of the trait were inherited from the sensitive parent).

Three QTLs for the late-sown set (Table 4). One QTL on chromosome 3 at about 100 cM seems to be common for early- and late-sown plants, although the peaks were 24 cM apart. Furthermore, a second QTL at chromosome 3 (11 cM), one at chromosome 7 (95 cM) and one at chromosome 8 (74 cM) were found in the early-sown set. In the late-sown plants, a strong QTL was detected at chromosome 5 (29 cM). All the QTLs for leaf area were due to the allelic contribution of the chilling-tolerant parent, with the exception of the QTL at chromosome 1 (181 cM) identified in the late-grown set.

For shoot dry weight of the seedlings sown early, two QTLs were found on chromosome 5 (90 cM) and 8 (39 cM). The increase in shoot dry weight was due to the allelic contributions of the chilling-tolerant and the chilling-sensitive parent, respectively (Table 4). The QTL on chromosome 5 was also found in the late-sown set. In plants sown late, a second QTL was detected on chromosome 3 at 86 cM with negative additivity.

The QTL analysis of traits obtained from the element analysis showed that C:N ratio was mostly influenced by the nitrogen content (Table 4); in early-sown plants the two QTLs at chromosomes 1 and 6 and in the late-sown plants the QTL at chromosome 9 were common for nitrogen content and C:N ratio. While in the early-sown plants a high C:N ratio and, respectively, a low nitrogen content, was inherited by the chilling-tolerant parent, the situation for the QTL was the opposite for the late-sown set.

Table 4: Main characteristics of QTLs with an LOD score >3.5 for area of the third leaf (LA; cm²), shoot dry weight (g plant⁻¹) carbon content (C%, g [C] g⁻¹ shoot dry weight), nitrogen content (N%, g [N] g⁻¹ shoot dry weight), and C:N ratio of the field experiment in 2003; see Table 3 for legends.

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<th>Trait</th>
<th>Chr</th>
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<th>LOD</th>
<th>$R^2$</th>
<th>Add</th>
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<td>3.93</td>
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Chr, chromosome number; cM, position of the peak of the QTL in centimorgans; Range, range of the QTL above the threshold LOD score; $R^2$, percentage of the phenotypic variance explained by genotype class at LOD peak; Add, Additivity (positive additivity = high values of the trait were inherited from the tolerant parent; negative additivity = high values of the trait were inherited from the sensitive parent).

Discussion

The photosynthetic performance, but also the shoot biomass accumulation, of the two parental lines differed considerably when the plants were exposed to low temperature early in the growing season. This difference disappeared when the plants developed under favorable temperature conditions. The photosynthetic apparatus of seedlings grown under cold conditions in the field showed the typical symptoms of growth at suboptimal temperature, namely low photosynthetic capacity and efficiency and low chlorophyll content, as demonstrated in several studies conducted under controlled environment conditions (Haldimann et al., 1996; Leipner et al., 1997; Nie et al., 1992) and in the field (Andrews et al., 1995; Leipner et al., 1999a). The regulation of the photosynthetic apparatus with respect to the fraction and the efficiency of open PSII reaction centers seemed to function similarly in both lines. However, seedlings of the chilling-sensitive line exposed to low growth temperature, were characterized by a higher yield for energy losses ($\Phi_{\text{NO}}$), which was not attributed to down-regulation; it indicated chilling-induced constitutive structural alterations of the photosynthetic apparatus.
The time of sowing and/or the climatic conditions during early seedling development also resulted in differential expression at several QTLs. Most of the identified QTLs derived from seedlings of the early-sown plots. Since the parental lines were selected for high or low PSII operating efficiency ($F_{v'/f_{m'}}$) at suboptimal growth temperature (Chapter 3; Fracheboud et al., 1999), this observation indicates that the selection method was efficient for the particular growth conditions. Moreover, the fact that the favorable alleles for most of the QTLs were inherited from the chilling-tolerant parent also shows the efficacy of the selection method.

Several genomic regions were found where different traits were under related genetic control. A major QTL for photosynthetic traits was located on chromosome 6 and was identified for the photosynthesis related parameters $F_{v'/f_{m'}}$, $F_{v}/F_{m}$ and $F_{o}$ in early-sown plants. This QTL was also found for $F_{v}/F_{m}$ in the late-sown set, but with a considerably smaller additivity, indicating its greater importance under cool conditions. A previous study, conducted with the same plant material grown under controlled conditions, revealed a QTL at the same position and for the same traits in plants grown at 15 °C, but not in plants developed at 25 °C (Chapter 4; Fracheboud et al., 2004). Under controlled low-temperature conditions, there was a pleitropic effect between the photosynthetic traits and the shoot dry weight at this locus, which led to the assumption that photosynthesis limits dry matter accumulation at suboptimal growth temperature. Seemingly, this was not the case in the field. This might be due to the higher light intensity under natural conditions, since a decrease in the maximum photosynthetic efficiency has a relatively stronger effect on photosynthetic activity at low light intensity than it has at high light intensity. Moreover, the probability that growth is source limited is much larger under low light than under high light intensity conditions. Taken together, changes in photosynthetic efficiency will have a smaller effect on growth at higher light intensity, as in the field, than at lower light intensity as is usual under growth chamber conditions. Nevertheless, in early-sown plants in the field, a QTL for the C:N ratio was detected at this locus, indicating the involvement of the quantum efficiency of PSII in the overall carbon assimilation. Furthermore, the QTL analysis revealed a weak QTL for shoot dry weight in the early-sown set at the end of chromosome 6 which was, however, with an LOD score of 1.9 below the threshold (data not shown). Since a QTL for leaf greenness was not detected at this locus, the molecular cause of the lower photosynthetic efficiency may be changes in the functioning of the photosynthetic apparatus or a feedback inhibition of photosynthesis, rather than a smaller amount of photosystems. This reduction in photosynthetic activity, however, seemed only partially to be the limiting factor for dry matter accumulation under field conditions. As discussed previously (Chapter 4; Fracheboud et al., 2004), an interesting candidate gene for this QTL might be agp2 coding for the small subunit of leaf ADP glucose pyrophosphorylase (Table 5). On chromosome 2 close to marker dupsr21, a common QTL was found for leaf greenness in early-sown seedlings and for $F_{v}/F_{m}$ in early- and late-sown seedlings, the latter, however, with low additivity. Moreover, QTLs for leaf greenness, carbon exchange rate and $F_{v'/f_{m'}}$ were identified at this position in this Chapter 4; Fracheboud et al., 2004) and in another population (Fracheboud et al., 2002) grown at suboptimal temperature under controlled conditions. The association between low chlorophyll content and reduction in the quantum efficiency of PSII might reflect a disturbance of the assembly of the photosynthetic apparatus, induced by low growth temperature. Aligning these results with the IBM2 Neighbors consensus genetic map (Maize Genetics and Genomics Database, www.maizegdb.org) revealed the presence of an interesting candidate gene at this locus:

### Table 5: Position of potential candidate genes in relation to the QTLs detected in this study and to common SSR markers from this population (ETH-population, ETH-DL3 × ETH-DH7), from the IBM2 2004 Neighbors map and the Pioneer composite 1999 map.

<table>
<thead>
<tr>
<th>Marker/gene/QTL*</th>
<th>Position (cM)</th>
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</tr>
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<tr>
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</tr>
<tr>
<td>bnlg121</td>
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<td>dupssr21</td>
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<tr>
<td>7QTL SPAD</td>
<td>110 (72-134)</td>
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<tr>
<td>myb2</td>
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<tr>
<td>sps2</td>
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<td>umc1930</td>
<td>104</td>
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</table>

* QTL position from 1, joint early; 2, joint late; 3, early 2003; 4, joint early; 5, joint late.
Moreover, a QTL for carbon exchange rate, some 3 in the late-sown seedlings (data not shown). A QTL for leaf greenness was identified close to the centromere of chromosome 10, close to umc1995, the nearest marker of another QTL for leaf greenness identified in the present study (Table 5).

For leaf greenness, leaf area and shoot dry weight a common QTL was located close to the centromere of chromosome 3. Similarly, several common QTLs for shoot dry weight and leaf area were found in maize seedlings grown in the greenhouse (Causse et al., 1995). With respect to the QTL at chromosome 3, however, high shoot dry weight was related to a small leaf area. Since the relative growth rate (RGR) reflects the product of net assimilation rate (NAR) and leaf area ratio (LAR) (Evans, 1972), one would expect that a QTL for carbon assimilation rate would be present at this location, which exhibits the same direction of additivity as for shoot dry weight, thus counteracting the smaller leaf area. Beside the QTL for leaf greenness, a QTL for $F_{v}/F_{m}$ with an LOD score of 2.4, which was, however, below the threshold, was identified close to the centromere of chromosome 3 in the late-sown seedlings (data not shown). Moreover, a QTL for carbon exchange rate, $F_{v}'/F_{m}'$, $F_{v}/F_{m}$ and leaf greenness, with the favorable allele inherited from the chilling-sensitive line (ETH-DL3) as in the present study, was revealed at this position in seedlings grown at optimal temperature in growth cabinets (Chapter 4; Fracheboud et al., 2004), supporting the involvement of photosynthesis in this QTL. The complexity of this QTL makes an explanation of its mode of action difficult. It is possible that the gene or the genes behind this QTL are involved in the control of cell division and, therefore, affect the biomass accumulation and the photosynthetic activity by altering the morphology of the leaf. A nearby located potential candidate gene for this mode of action would be myb2, which codes the cell division control protein 5 (CDC5) (Table 5). Alternatively, changes in photosynthetic performance could be the primary cause, which consequently would affect shoot growth and leaf greenness and area. According to the IBM2 Neighbors consensus genetic map, a potential candidate gene, which is located near the identified QTL and which may explain the pleiotropic effect at this locus by the latter mode of action is sps2, a gene for sucrose phosphate synthase (SPS) (Table 5). The effect of SPS activity on photosynthesis is well documented for C3-plants overexpressing SPS (for a review see Huber and Huber, 1996). Moreover, an effect of SPS activity on chlorophyll content was observed in SPS transformants of Arabidopsis thaliana (Strand et al., 2003). However, its effect on photosynthesis in C4 plants has not been extensively studied. It may be of less importance since a short-term feedback inhibition of SPS by sucrose seems not to occur in maize leaves (Lunn and Hatch, 1997). If the chlorophyll content is indirectly regulated by the activity of enzymes involved in carbon assimilation, as also found for Rubisco in the C4 plant Flaveria bidentis (Kubien et al., 2003), then one would expect that all major QTLs for photosynthetic activity are associated with QTLs for leaf greenness. Clearly, this was not the case regarding the QTL at the end of chromosome 6, indicating that changes in photosynthetic activity does not necessarily affect the chlorophyll content. Only one QTL was identified for shoot dry weight which was stable in all the investigated environments. This QTL was located on chromosome 5 at about 90 cM, and the favorable allele was inherited from the chilling-tolerant parent. It was also detected for shoot dry weight in seedlings sown in the autumn which developed under cold conditions (data not shown). The QTL at chromosome 5 was not related to any other trait; and it was not identified under controlled conditions either for this trait or for any of the other traits (Chapter 4; Fracheboud et al., 2004). According to the IBM2 Neighbors consensus genetic map, this QTL is located near the nmr2 gene, whose product is NAD(P)H nitrate reductase (Table 5). There is a strong indication that nitrate reductase is important for this QTL as shown by Hirel et al. (2001) for another segregating population. In the latter population, a QTL for nitrate reductase activity and nitrate content was identified in the same region of chromosome 5 when the map was aligned to the IBM2 Neighbors consensus map. Since this part of the chromosome has no influence on the C:N ratio, the gene or the gene cluster behind the QTL at chromosome 5 might influence the shoot dry weight through the availability of nitrogen for growth. On the other hand, if carbon limits growth, then a QTL for photosynthesis or leaf area would be expected at this locus, which was not the case. The hypothesis that the activity of nitrate reductase affects the shoot dry weight is also supported by the high correlation between nitrate reductase activity at the seedling stage and final plant biomass of European maize cultivars (Feil et al., 1993). Together with previous results obtained under controlled conditions (Chapter 4; Fracheboud et al., 2004), it is evident that the main QTLs involved in the functioning of the photosynthetic apparatus at low temperature are stable in cold environments. Furthermore, the QTL analysis indicated that the chilling-induced reduction in photosynthetic activity
can be caused by perturbation in the assembling of the photosynthetic apparatus, as well as by reduced enzyme activity down-stream of the photosynthetic light reaction. The former was implied by the co-location of QTLs for photosynthetic efficiency and leaf greenness, the latter was indicated by QTLs which were found only for photosynthetic efficiency but not for leaf greenness. The functioning of the photosynthetic apparatus in plants developed at low temperature in the field, however, did not noticeably affect biomass accumulation, in contrast to the findings under controlled conditions. Nitrogen assimilation is probably more important for seedling growth in the field, because a larger leaf area, which may also positively influence growth, did not affect biomass accumulation of the investigated material either. The comparison of the QTLs with QTLs of similar traits at the seedling stage in two other mapped populations (Causse et al., 1995; Fracheboud et al., 2002) revealed some agreement. Similarities between mapped populations were found when the populations were grown under similar conditions and when traits characterizing the physiology of the photosynthetic apparatus were considered (Chapter 4; Fracheboud et al., 2004), indicating that the genetic basis of the chilling tolerance of photosynthesis is similar in different maize germplasms.
Chapter 6

Genetic analysis of chilling-related seedling traits reveals little relevance for yield in maize*

Prolonged low temperature phases and short-term cold spells often occur in spring during the crucial stages of early maize (Zea mays L.) development. The effect of low temperature-induced growth retardation at the seedling stage on final yield is poorly studied. Therefore, the aim was to identify genomic regions associated with morpho-physiological traits at flowering and harvest stage and their relationship to previously identified QTLs for photosynthesis and morpho-physiological traits from the same plants at seedling stage. Flowering time, plant height and shoot biomass components at harvest were measured in a dent mapping population for cold tolerance studies, which was sown in the Swiss Midlands in early and late spring in two consecutive years. Early sown plants exhibited chilling stress during seedling stage while late sown plants grew under favorable conditions. Significant QTLs, which were stable across environments, were found for plant height and for the time of flowering. The QTLs for flowering were frequently co-localized with QTLs for plant height or ear dry weight. The comparison with QTLs detected at seedling stage revealed only few common QTLs. A pleiotropic effect was found on chromosome 3 which revealed that a good photosynthetic performance of the seedling under warm conditions had a beneficial effect on plant height and partially on biomass at harvest. However, a high chilling tolerance of the seedling seemingly had an insignificant or small negative effect on the yield.

Introduction

Maize (Zea mays L.) is a crop from the (sub) tropics but was cultivated at higher geographic latitudes in America already before the New World was discovered. Early North-American flint populations were introduced to Central Europe shortly afterwards (Rebourg et al., 2003; Revilla et al., 2003). However, maize of the dent type is still characterized by a low chilling tolerance (Dolstra et al., 1988). In particular, it is essential to maximize the yield potential of maize under a combination of suboptimal temperature and moderately high light intensity. Adaptation to such marginal growth conditions has been successful due to breeding for early maturing maize plants, compromising between the risk of yield loss and an acceptable level of yield gain (Stamp, 1986). In the temperate regions, suboptimal temperatures occurring during spring affect seedling establishment and photosynthesis (Leipner et al., 1999a; Stirling et al., 1991). They preclude early sowing, so that at high-latitude, flowering and grain filling occur when both radiation and temperature are declining, which can affect yield potential (Otegui and Bonhomme, 1998). Furthermore, drought occurs frequently around flowering which is a critical period for grain set (c.f. Hall et al. 1981). Therefore, maize with improved chilling tolerance could be sown earlier, resulting in a better fit between crop cycle and availability of natural resources. While the beneficial effects of a high chilling tolerance on early vigor is widely recognized its effect on the further plant development and finally on the yield is less clear. Few studies indicate that seedling development has indeed an effect on grain yield. For example, transplanting experiments in southern England revealed that in cool years yield was higher when seedlings were raised for some days under more favorable conditions in greenhouses as compared to field-sown plants (Dale and Drennan, 1997). Recently, Chenu et al. (2007) showed that organ growth and development was affected during the whole plant cycle by low temperature although the cold period was limited to the first stages of maize development. Therefore, improvement of chilling tolerance of maize seedlings is a desirable aim for a more stable and higher crop yield. However, Greaves (1996) points out that improving a single suboptimal temperature tolerance trait per se may not influence the yield performance of a particular genotype in a temperature limiting environment. Rather the combination of multiple tolerance traits might allow a better performance throughout the growing season resulting finally in a higher yield. In order to unravel the interaction of such complex

* based on the following publication:
traits, quantitative trait loci analysis has become a useful tool for plant physiologists and breeders (Vreugdenhil et al., 2007). Extensive genome mapping of maize based on DNA markers has been accomplished. These maps and their associated technology have been used successfully for a number of quantitative trait loci (QTL) analyses. A number of papers deal with the chilling tolerance of maize seedlings (Fracheboud et al., 2002; Fracheboud et al., 2004; Hund et al., 2005; Jompuk et al., 2005; Pimentel et al., 2005; Presterl et al., 2007). Once the significance of these QTLS for chilling tolerance has been verified, it should become possible to pyramiding the favorable alleles into elite germplasm through marker-assisted selection (MAS).

Using a method based on chlorophyll fluorescence measurements, we have developed a set of inbred lines with contrasting chilling tolerance of photosynthesis (Chapter 3; Fracheboud et al., 1999). Since these lines were selected solely on the basis of chilling tolerance of photosynthesis there are a unique material to identify genetic loci associated with chilling tolerance of photosynthesis by quantitative trait loci (QTL) analysis. In previous studies, we have examined this material in respect to chilling tolerance at the seedling stage under controlled conditions (Chapter 4; Fracheboud et al., 2004) and in the field (Chapter 5; Jompuk et al., 2005). These studies revealed major QTLS for chilling tolerance of photosynthesis which were stable across cold environments. Furthermore, consistent QTLS for biomass accumulation and for photosynthesis under warm conditions were identified.

Aim of this study was to elucidate the relevance of chilling tolerance of seedlings for yield related traits. Studies investigating the effect of chilling tolerance of maize seedlings on the grain yield were conducted with individual genotypes (Mock and McNeill, 1979) or hybrids (Revilla et al., 2000). So far, there are no studies in term of quantitative trait loci (QTL) for chilling stress during seedling stage and its relation to the biomass yield at harvest. We hypothesized that if chilling tolerance at seedling stage has a beneficial effect on maize yield then common QTLS for chilling tolerance at seedling stage and for yield related traits at harvest will be found. In order to corroborate this, a QTL analysis was conducted in a segregating F2:3 population grown in the field; flowering and yield related traits were determined. The QTLS for these traits were compared with QTLS for photosynthesis related traits and for growth parameters obtained from the same plants during early development under low temperature (Chapter 5; Jompuk et al., 2005).

### Materials and methods

#### Plant material

A dent maize (Zea mays L.) mapping population in the F2:3 generation, consisting of 226 families derived from the cross ETH-DH7 and ETH-DL3, was used in the present QTL experiment (for details see Fracheboud et al. 2004). The source material was derived from the actual breeding population of the Swiss DSP (Delley Samen und Pflanzen AG, Delley, Switzerland). The parental lines of the population are characterized by a contrasting chilling-tolerance of photosynthesis and were obtained by divergent selection from a Swiss dent maize breeding population using the chlorophyll fluorescence parameter Fv'/Fm' (PSII operating efficiency) as selection criteria (Chapter 3; Fracheboud et al., 1999).

#### Field experiments

The field experiments were conducted at the experimental station of the Institute of Plant Sciences of the ETH in Eschikon near Zurich, Switzerland (47° 26’ N, 8° 40’ E, 550 m a.s.l.). In 2002, the F2:3 population was sown on 26 April (early sowing) and 24 May (late sowing). In 2003, sowing was done on 14 April (early) and 15 May (late) (see also Chapter 5 and Jompuk et al. 2005). Fertilization was applied according to good agricultural practice. The N fertilization (NH4NO3) was 115 kg ha-1 and was split into two applications at the 3- and 6-leaf stage. Weeds were controlled by a combination of atrazine, sulcotriion and nicosulfuron.

The experiments were over-sown by machine and thinned to the final plant number of 13 per m2 after emergence. The experimental unit was a single-row plot with 50 plants, 5 m long and 0.75 m between the rows. Trials of the 226 F2:3 lines were conducted using an alpha (0,1) lattice design with 23 blocks per replication (Patterson and Williams, 1976) and two replications for each sowing date. Each replication was bordered by two rows of a mixture of the F2:3 lines. In each experimental unit, the first two plants were not used for measurements. The soil was a Eu tric Cambisol (FAO classification) with a clay loam (CL) texture and a low content of organic matter (3%) (Richner et al., 1996). Meteorological data were recorded at 15-min intervals, 2 m above the soil surface.

#### Morpho-physiological traits

Female and male flowering was defined as the number of days from sowing to the day when half of the plants from each experimental unit showed the female flower and began to shed pollen, respectively. The plant height was determined from ten plants per experimental unit before harvest. The dry weight at final harvest was determined from ten plants. The
shoot of the plants was cut at ground level; ears without husk leaves and the rest of the plants (straw) were separately shopped by machine. Aliquots of samples were oven dried at 90 °C for at least 72 h.

**Statistical analysis and QTL mapping**

The broad-sense heritability ($h^2$) was estimated over the two years as described by Hallauer and Miranda (1981). Confidence intervals of heritability were calculated according to Knapp et al. (1985). As input for the QTL analyses, adjusted means of the trait values were calculated by the program Alphagen version 2.3 (Scottish Agricultural Statistics Service, Edinburgh). The QTL analyses were performed using the program QTL Cartographer version 1.17b (Basten et al., 2005). The method of composite interval mapping, model 6 of the Zmapqtl program module of QTL Cartographer, was deployed for mapping the QTLs (Basten et al., 1994). Joint analyses of the phenotypic data from multiple environments by composite interval mapping was computed with the JZmapqtl procedure of QTL Cartographer (Jiang and Zeng, 1995). In both cases, the genome was scanned at 2-cM intervals and the window size was set at 30 cM. Co-factors were chosen using the forward-backward method of step-wise regression at $p(F_{in}) = p(F_{out}) = 0.05$. The presence of a QTL was declared significant when the likelihood of odds (LOD) value was higher than 3.5 for a single-environment analysis and higher than 6.0 for the joint analysis of four environments. These values correspond to a Type-I error rate ($\alpha$ level) of 0.021, assuming that all the chromosome arms segregate independently. Additive effects of the detected QTLs were estimated by the Zmapqtl procedure of QTL Cartographer; the $R^2$ value (coefficient of determination) from this analysis indicated the percentage of phenotypic variance explained by marker genotypes at the locus.

**Results**

**Environmental effects**

The two experimental years were characterized by different climatic conditions throughout the experimental phases (Figure 1). In both years, seedlings of the early-sown set developed under much lower temperature than the late-sown plants (see also Chapter 5 and Jompuk et al., 2005). In general, the growing season of 2002 was cooler than 2003. The average daily temperature of July and August 2002 and, in particular, of September 2002 was below the longstanding average (MeteoSchweiz, www.meteoschweiz.ch). However, the temperature in June 2002 was considerably above this average. The climatic conditions of 2003 were characterized by very high temperatures and low precipitation. In 2003, the warmest June since beginning of recording in 1753 was measured for Switzerland. Furthermore, the meteorological summer of 2003 was one of the ten driest in the region of Eschikon (station Zurich) since 1900 (MeteoSchweiz, www.meteoschweiz.ch). The time from sowing until flowering reflected the different climatic conditions. In 2003, the time until male flowering was in average 12 days (early sowing) and 6 days (late sowing) shorter than in 2002 (Table 1). The time until male flowering of late sown $F_{2:3}$ plants was 14 days (2002) and 7 days (2003) shorter than the one of early sown plants. The contrasting climatic conditions were also reflected in the time until maturation, which was about 10 days shorter in early sown plants in 2003 than in 2002. In 2002, plants of the late-sown set had to be harvested before the black layer of the seeds was fully developed. Consequently, shoot dry weight was considerably lower in the late sown set of 2002 as compared to the other sets (Table 1). Differences between the years were also found in respect to the
proportion of the ear weight on the shoot weight; it was significantly lower in 2003 than in 2002 (data not shown). The high influence of the climatic conditions on the time until flowering as well as on the ear and shoot dry weight was also reflected in a low heritability for these traits (Table 1). The plant height was characterized by a high heritability and, consequently, there was only a small effect of years and sowing dates on plant height.

Quantitative trait loci analysis

The joint QTL analysis, combining data of the four environments, revealed several chromosomal regions, which were associated with the time of flowering, plant height and biomass at harvest. For the date of male flowering, two QTLs were detected (Table 2). These QTLs were located on chromosomes 1 (13 cm) and 9 (50 cm) and were found for the date of female flowering as well. Both QTLs were characterized by high LOD scores in 2002 but not in 2003, with the exception of the QTL at chromosome 9 which was significant in the early set of 2003. Plants, which inherited the chromosomal region from ETH-DH7 at these positions, flowered later in 2002 but earlier in 2003. For female flowering date, three additional QTLs were detected on chromosomes 5 (72 cm) and 7 (27 and 110 cm). Again, these QTLs were highly significant in 2002 but below the threshold in 2003.

Table 1: Summary statistics including means, variances ($\sigma^2_x$ and $\sigma^2_y$) and heritability ($h^2$) for flowering traits, plant height at flowering and straw, ear, and shoot dry weight at harvest of the F$_2$$_3$$_3$ families.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Year</th>
<th>Env.</th>
<th>Mean ± SD Min. Max.</th>
<th>$\sigma^2_x$</th>
<th>$\sigma^2_y$</th>
<th>$\sigma^2_x$ $\sigma^2_y$</th>
<th>$h^2$ % C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male flowering</td>
<td>2002</td>
<td>E</td>
<td>94.9 ± 2.1</td>
<td>86</td>
<td>103</td>
<td>81.4 ± 2.9</td>
<td>75</td>
</tr>
<tr>
<td>days after sowing</td>
<td>2003</td>
<td>E</td>
<td>82.9 ± 1.5</td>
<td>77</td>
<td>87</td>
<td>76.0 ± 1.0</td>
<td>74</td>
</tr>
<tr>
<td>Female flowering</td>
<td>2002</td>
<td>E</td>
<td>97.8 ± 2.3</td>
<td>92</td>
<td>104</td>
<td>83.3 ± 2.7</td>
<td>87</td>
</tr>
<tr>
<td>days after sowing</td>
<td>2003</td>
<td>E</td>
<td>84.8 ± 1.6</td>
<td>80</td>
<td>89</td>
<td>77.5 ± 1.2</td>
<td>75</td>
</tr>
<tr>
<td>Plant height</td>
<td>2002</td>
<td>E</td>
<td>207.5 ± 16.7</td>
<td>166</td>
<td>249</td>
<td>212.6 ± 16.1</td>
<td>171</td>
</tr>
<tr>
<td>cm</td>
<td>2003</td>
<td>E</td>
<td>199.7 ± 18.0</td>
<td>149</td>
<td>245</td>
<td>214.7 ± 19.1</td>
<td>167</td>
</tr>
<tr>
<td>Shoot dry weight</td>
<td>2002</td>
<td>E</td>
<td>148.7 ± 25.0</td>
<td>87</td>
<td>250</td>
<td>119.0 ± 20.5</td>
<td>48</td>
</tr>
<tr>
<td>g Plant$^{-1}$</td>
<td>2003</td>
<td>E</td>
<td>147.0 ± 24.9</td>
<td>72</td>
<td>245</td>
<td>153.1 ± 41.6</td>
<td>68</td>
</tr>
<tr>
<td>Straw dry weight</td>
<td>2002</td>
<td>E</td>
<td>74.6 ± 15.0</td>
<td>26</td>
<td>142</td>
<td>61.3 ± 12.3</td>
<td>24</td>
</tr>
<tr>
<td>g Plant$^{-1}$</td>
<td>2003</td>
<td>E</td>
<td>80.5 ± 17.4</td>
<td>35</td>
<td>155</td>
<td>82.8 ± 17.1</td>
<td>34</td>
</tr>
<tr>
<td>Ear dry weight</td>
<td>2002</td>
<td>E</td>
<td>74.1 ± 14.5</td>
<td>36</td>
<td>151</td>
<td>57.7 ± 12.2</td>
<td>21</td>
</tr>
<tr>
<td>g Plant$^{-1}$</td>
<td>2003</td>
<td>E</td>
<td>66.3 ± 12.4</td>
<td>15</td>
<td>156</td>
<td>70.5 ± 27.0</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 2: Main characteristics of QTLs for the date of male and female flowering, plant height and shoot, straw and ear dry weight of maize sown early (E) or late (L) in the year 2002 and 2003 with an LOD score above a threshold of 6.0 for the joint analysis.

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>Male flowering</td>
<td>2002</td>
<td>9 50</td>
<td>38-269</td>
<td>phi227</td>
<td>6.72</td>
<td>6.17</td>
<td>3.36</td>
<td>3.53</td>
<td>0.81</td>
<td>0.02</td>
<td>4.3</td>
<td>4.7</td>
<td>2.9</td>
<td>0.1</td>
<td>0.55</td>
<td>0.81</td>
<td>-0.12</td>
<td>-0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>days after sowing</td>
<td>2003</td>
<td>9 50</td>
<td>38-269</td>
<td>phi227</td>
<td>5.17</td>
<td>5.00</td>
<td>3.52</td>
<td>3.67</td>
<td>0.79</td>
<td>0.02</td>
<td>5.55</td>
<td>6.66</td>
<td>-0.46</td>
<td>-0.09</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female flowering</td>
<td>2002</td>
<td>9 50</td>
<td>38-269</td>
<td>phi227</td>
<td>12.13</td>
<td>9.73</td>
<td>7.67</td>
<td>7.22</td>
<td>0.32</td>
<td>0.07</td>
<td>9.5</td>
<td>3.5</td>
<td>1.0</td>
<td>0.2</td>
<td>0.87</td>
<td>0.77</td>
<td>-0.03</td>
<td>-0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>days after sowing</td>
<td>2003</td>
<td>9 50</td>
<td>38-269</td>
<td>phi227</td>
<td>7.56</td>
<td>5.78</td>
<td>2.61</td>
<td>2.54</td>
<td>0.62</td>
<td>1.98</td>
<td>6.5</td>
<td>8.0</td>
<td>1.8</td>
<td>7.1</td>
<td>-0.14</td>
<td>0.10</td>
<td>0.28</td>
<td>0.37</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Plant height</td>
<td>2002</td>
<td>1 50</td>
<td>0-66</td>
<td>bnlg1112</td>
<td>13.24</td>
<td>9.64</td>
<td>6.19</td>
<td>5.87</td>
<td>4.36</td>
<td>5.46</td>
<td>11.2</td>
<td>2.5</td>
<td>8.0</td>
<td>6.2</td>
<td>6.99</td>
<td>3.36</td>
<td>5.80</td>
<td>4.69</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cm</td>
<td>2003</td>
<td>1 50</td>
<td>0-66</td>
<td>bnlg1112</td>
<td>10.05</td>
<td>7.53</td>
<td>5.00</td>
<td>4.96</td>
<td>3.81</td>
<td>4.76</td>
<td>9.6</td>
<td>1.8</td>
<td>7.8</td>
<td>7.1</td>
<td>9.09</td>
<td>6.48</td>
<td>5.80</td>
<td>4.69</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Shoot DW</td>
<td>2002</td>
<td>6 56</td>
<td>27-147</td>
<td>bnlg249</td>
<td>7.69</td>
<td>6.17</td>
<td>7.42</td>
<td>6.22</td>
<td>0.21</td>
<td>0.30</td>
<td>6.8</td>
<td>6.3</td>
<td>0.5</td>
<td>0.6</td>
<td>-0.85</td>
<td>-1.04</td>
<td>-0.02</td>
<td>-0.10</td>
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<td></td>
</tr>
<tr>
<td>g Plant$^{-1}$</td>
<td>2003</td>
<td>6 56</td>
<td>27-147</td>
<td>bnlg249</td>
<td>7.69</td>
<td>6.17</td>
<td>7.42</td>
<td>6.22</td>
<td>0.21</td>
<td>0.30</td>
<td>6.8</td>
<td>6.3</td>
<td>0.5</td>
<td>0.6</td>
<td>-0.85</td>
<td>-1.04</td>
<td>-0.02</td>
<td>-0.10</td>
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<tr>
<td>Straw DW</td>
<td>2002</td>
<td>6 56</td>
<td>27-147</td>
<td>bnlg249</td>
<td>7.69</td>
<td>6.17</td>
<td>7.42</td>
<td>6.22</td>
<td>0.21</td>
<td>0.30</td>
<td>6.8</td>
<td>6.3</td>
<td>0.5</td>
<td>0.6</td>
<td>-0.85</td>
<td>-1.04</td>
<td>-0.02</td>
<td>-0.10</td>
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</tr>
<tr>
<td>g Plant$^{-1}$</td>
<td>2003</td>
<td>6 56</td>
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<td>6.17</td>
<td>7.42</td>
<td>6.22</td>
<td>0.21</td>
<td>0.30</td>
<td>6.8</td>
<td>6.3</td>
<td>0.5</td>
<td>0.6</td>
<td>-0.85</td>
<td>-1.04</td>
<td>-0.02</td>
<td>-0.10</td>
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</tbody>
</table>

Env. = Environment; E = Early sowing; L = Late sowing; SD = Standard deviation; ** = non significant difference; *** = significance difference at $P < 0.05$; **** = significance difference at $P < 0.01$; ***** = significance difference at $P < 0.001$.

Chr = Chromosome number, cm = Position of the peak of the QTL in centimorgan, Range = range was defined by the positions on the chromosome where the LOD score at the QTLs peaks decreased by half, Joint = LOD score in the joint analysis of the two sowing in the years 2002 and 2003, $Q \times E$ = LOD score value for QTL-environment interaction in the joint analysis, $R^2$ = Percentage of the phenotypic variance explained by genotype class at LOD peak, Add = Additivity.
For plant height, a large number of QTLs was detected. The high heritability for this trait was reflected in a high stability of most of the QTLs across environments (Table 2). In particular, the QTL on chromosome 3 (108 cM) was very stable across sowing dates and years. Four more QTLs, which were little affected by the environment and which were characterized by high LOD scores were localized on chromosome 1 (4 and 105 cM), beginning of chromosome 5 and on chromosome 6 (56 cM). The allele of ETH-DL3 was responsible for an increased plant height concerning the QTLs on chromosomes 1 (105 cM), 3 (0 and 108 cM), 5 and 8.

Shoot dry matter at harvest was dissected in two parts, ear dry weight and straw dry weight. The number of QTLs for the traits at harvest was rather low. The joint analysis revealed one QTL, on chromosome 7 at about 31 to 36 cM, which was detected for shoot weight and its components straw and ear dry weight; it was strongest in the late sown set of 2003 (Table 2). The allelic contribution for high shoot, straw and ear dry weight at this QTL came from ETH-DH7, the cold-tolerant parent.

Since climatic conditions were considerably different between the four environments, QTL analyses at individual environments were conducted as well. Additionally to the QTLs revealed by the joint analyses, further QTLs were found in particular for trait data from 2002. For this data set, QTLs were identified for straw dry weight on chromosome 2 (about 130 cM), for ear, straw and shoot dry weight on chromosome 9 (49-88 cM) and for shoot dry weight on the beginning of chromosome 10 (Figure 2). Furthermore, a common QTL was identified for shoot, straw and ear dry weight at chromosome 6 (50 cM) in the early-sown plants of 2002 whose favorable allele was inherited from ETH-DH7. In 2003, QTLs were found for shoot and straw dry weight (early-sown in 2003) on chromosome 2 (235 cM) and for ear and shoot dry weight (late-sown in 2003) at 84 cM on chromosome 10 (Figure 2).

Comparison with QTLs for seedling traits

In order to examine the effect of seedling vigor on the yield, the QTL data of the present study were compared with those identified at the seedling stage in the same experiments (Chapter 5; Jompuk et al., 2005). Due to high \( Q \times E \) interaction for most of the QTLs, the QTL analyses were conducted for individual environments. These analyses revealed the presence of overlapping QTLs on chromosomes 2, 3 and 10 (Figure 2). The QTL for the PSII operating efficiency \( \left( \frac{F_{q'}}{F_{m'}} \right) \) and for leaf greenness (SPAD) identified previously near the centromere of chromosome 2 in seedlings of the early sown set (Jompuk et al. 2005), mapped close to a QTL for straw dry weight in 2002. However, the additivity of this QTL at seedling stage was positive while it was negative for straw dry weight. Similarly, an overlapping QTL with contrasting additivity was found in the late sown set in 2003 on chromosome 10 for SPAD at seedling stage (positive additivity) and ear and shoot dry weight (negative additivity). The region close to marker \( mma0022 \) on chromosome 3 harbored QTLs for leaf greenness...
and shoot dry weight of the seedlings as well as for straw and shoot dry weight at harvest in the late sown plants in 2002 and the early sown plants in 2003. At this locus, the additivity of all QTLs was negative, meaning that the favorable allele was inherited from the chilling-sensitive parent ETH-DL3.

Discussion

The biomass accumulation of maize plants was strongly affected by the climatic conditions during growth. In 2002, which was characterized by lower temperatures than 2003, seeds of late-sown plants were not fully mature (physiological maturity) before the first frost. Consequently, the biomass accumulation was lower in the late sown set compared to the early sown plants. Another factor, which influenced the biomass accumulation, was the time of flowering. Co-localizations of QTLs for ear and straw dry matter with QTLs for the date of flowering were found. The signs of additivity clearly demonstrated that, as it can be expected, late flowering was beneficial for a higher biomass due to a longer vegetative growth. Similarly, Moreau et al. (2004) frequently found co-localizations of silking date and grain yield in a maize F$_{2}$:$_{3}$ population grown under a large range of environmental conditions. However, other QTLs for flowering dates were associated with QTLs for plant height but not with QTLs for dry matter; some QTLs for flowering dates did not show any pleiotropic effect. This observation indicates that late flowering does not directly cause higher biomass rather it seems that both traits can be independently controlled by the same gene or genomic region.

The main focus of this study was to answer the question whether a good chilling tolerance during seedling stage has a beneficial effect on the biomass at harvest. A significant correlation between grain yield and dry weight of seedlings, which were exposed to low temperature, was found in a set of maize inbred lines (Mock and McNeill, 1979). However, results of Revilla et al. (2000) indicated that significant differences among hybrids for early vigor under low temperature conditions were not correlated with grain yield. The comparative analysis of QTLs identified in seedlings grown under chilling conditions in the field (Chapter 5; Jompuk et al., 2005) with the QTLs found at harvest indicated that photosynthesis related traits and morpho-physiological traits, like shoot dry weight, area of the third leaf, carbon and nitrogen concentration and C:N ratio, at seedling stage did not result in an increased biomass at harvest. Concerning two of the overlapping QTLs it seems even that higher leaf greenness and higher photosynthetic efficiency had a negative effect on the final biomass. What is the reason of this poor relationship between chilling tolerance of the seedling and the final biomass? The analysis of the QTL on chromosome 3 might shed some light into this question. In the immediate vicinity of marker mmc0022, a QTL was found for plant height and for ear dry weight. The position of this QTL was co-localized with a QTL for photosynthesis-related traits detected in the late-sown plants of the mapping population at the seedling stage (Chapter 5; Jompuk et al., 2005). This QTL was also found in seedlings grown at 25 °C in growth chambers (Chapter 4; Fracheboud et al., 2004). At the seedling stage too, the allele for good photosynthetic performance under warm conditions was inherited from the chilling-sensitive parent. This observation indicates that a good photosynthetic performance under optimal conditions increases yield. The mentioned chromosomal region of chromosome 3 harbored also a QTL for the area of the third leaf (Chapter 5; Jompuk et al., 2005), those sign of additivity revealed that the higher photosynthetic activity was associated with a smaller leaf area at this locus. Since at this position no QTL with contrasting additivity was found for photosynthetic performance under chilling conditions, high photosynthesis under warm conditions seems not to have a drawback on the photosynthesis in the cold.

Another reason of the poor correlation between QTLs at maturity and QTLs for chilling tolerance at seedling stage might be the weather conditions during the later plant development which might have a predominant effect. In the year 2002, a hail storm during June drastically diminished the leaf area of the maize plants. Plants with a better early growth were at least as strongly affected as plants with a poor early vigor with respect to the loss of leaf area. The rest of the growing season in 2002 was characterized by rather low temperatures. The QTLs, which were found for yield related traits solely in 2002, were potentially involved in the late cold tolerance. Since the genetic control of cold tolerance seems to be independent at different growth stages (Revilla et al., 2000) it might explain why these QTLs were not co-localized with QTLs for cold tolerance of photosynthesis at seedling stage. The year 2003 was characterized by severe drought stress and, therefore, this stress factor might have had a stronger effect on the yield than the chilling stress during seedling establishment. For the early sown set in 2003, plants of the F$_{2}$:$_{3}$ population segregated in leaf rolling which is a good indicator for the severity of the drought stress (Bänziger et al., 2000). Two QTLs were found for this trait, on chromosomes 1 (79 cM, LOD = 5.17) and 3 (108 cM, LOD = 4.05). In both cases, there was a pleiotropic effect on plant height, which was characterized by a contrasting additivity, meaning that tall plants suffered less from drought stress than short ones did. A positive relationship between root length and plant height was found i.e. during root selection in hybrid maize.
breeding (Rady, 1996). Since no co-localization of QTLs for leaf rolling and for ear dry weight were found it seems less likely that genotypic differences in drought tolerance, which might be due to differences on rooting depth, had an influence on the yield in the year 2003.

In conclusion, the QTL analysis revealed that high chilling tolerance of photosynthesis at the seedling stage did not have a large effect on the later yield of the maize mapping population in our study. Other morpho-physiological traits, which in addition described the seedling vigor such as seedling shoot dry weight, leaf area and nitrogen concentration, did not to give clues to the later biomass accumulation as well. The present results indicate that the performance of the seedlings under chilling conditions in respect to photosynthesis and growth may not have a large effect on the biomass accumulation during later growth stages. The capacity of compensation in maize seems to be high and the conditions during later growth phases might be more determining for biomass accumulation. This is in accordance with earlier studies indicating that flowering and early seed development are the critical developmental period for determining maize grain yield (Classen and Shaw, 1970; Shaw, 1988). Although, an effect of early vigor on grain yield seems to depend largely on later growth conditions, a sufficient chilling tolerance of the maize seedling is beneficial, because of an improved capability of seedling to compete with weeds and to avoid fungal infection (Messiaen et al., 1976; Messiaen et al., 1977) and a lower risk of soil erosion and nitrate leaching.
Chapter 7

Genetic analysis of chlorophyll fluorescence parameters in maize seedlings grown under different excitation pressures*

The chilling sensitivity of maize (Zea mays L.), which is associated with a perturbed development of the photosynthetic apparatus when seedlings grow at suboptimal temperature, shows genetic variation. Several quantitative trait loci (QTLs) for chilling tolerance of photosynthesis have been identified elsewhere. In this study, QTL analyses of chlorophyll fluorescence parameters of a segregating F$_{2:3}$ population were conducted in order to unravel the interactions among the chlorophyll fluorescence parameters and to better understand the role of excitation pressure in the expression of the major QTLs for chilling tolerance of photosynthesis. Seedlings of the mapping population were grown at optimal and suboptimal temperature at moderate light intensity as well as at optimal temperature and high light intensity. Chlorophyll fluorescence induction was analyzed under the respective growth conditions and in dark-adapted plants. The joint QTL analysis across the three tested environments identified three genomic regions, which were involved in the expression of most of the studied chlorophyll fluorescence parameters. Of these QTLs, the QTL on chromosome 3 was expressed only at optimal temperature, independent of the light intensity, and the QTL on chromosome 1 was expressed only at suboptimal temperature. The QTL on chromosome 6 was expressed in all environments, but the greater allelic effect at suboptimal and optimal temperature and high light intensity indicates the role of excitation pressure in its expression, in particular for the maximum quantum efficiency of PSII photochemistry ($F_v / F_m$). In-depth analysis of these QTLs revealed that genetic differences in the PSII operating efficiency ($F_q' / F_m'$) were caused by genetic differences in the PSII maximum efficiency ($F_v' / F_m'$) and/or the PSII efficiency factor ($F_q' / F_v'$), which reflects the redox state of QA. The results suggest that $F_q' / F_m'$ is controlled mainly by $F_v' / F_m'$ under high excitation pressure, while components downstream of $P_{680}$ modulates $F_q' / F_m'$ under low excitation pressure. Genetic alterations of $F_v / F_m$ were attributed to constitutive damage of the PSII reaction center and, at suboptimal temperature, to persistent down-regulation.

Introduction

As a crop of (sub)tropical origin, maize (Zea mays L.) is very sensitive to low temperature, in particular during the transition from heterotrophic to autotrophic growth. Among the various effects of chilling on the maize seedling, the disturbed development of the photosynthetic apparatus seems to be of particular importance (Leipner and Stamp, 2009). These effects are more pronounced when seedlings develop under high light intensity, especially in chilling-sensitive genotypes (Haldimann, 1998). A considerable part of this research on the chilling tolerance of maize was done by means of chlorophyll fluorescence analysis, a powerful method for studying the plant's response to environmental stress factors (Fracheboud and Leipner, 2003). In particular, the saturation pulse method is very valuable, and several chlorophyll fluorescence parameters have been identified, which characterize the status of PSII (Maxwell and Johnson, 2000). Among these parameters, the PSII operating efficiency ($F_q' / F_m'$, formerly referred to as $\Delta F / F_m'$, $\Phi_{PSII}$ or Genty parameter) has received much attention, because it is an estimate of the quantum yield of linear electron transport through PSII and, under non-photorespiratory conditions, it is linearly related to the quantum efficiency of carbon fixation (Genty et al., 1989). Moreover, it does not require dark adaptation, making it a very attractive parameter for screening, with which plants of varying stress tolerance can be characterized (e.g. maize seedling for chilling tolerance; Fracheboud et al., 1999). The $F_q' / F_m'$ parameter is mathematically the product of $F_v' / F_m'$, which describes the PSII maximum efficiency of light-adapted leaves, and the PSII efficiency factor $F_q' / F_v'$ (formerly named $q_P$). The PSII efficiency factor is nonlinearly related to the redox state of QA (for a review see Baker, 2008). Although $F_q' / F_m'$ can be independently affected by $F_v' / F_m'$.

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Leipner, J., Jompuk, C., Fracheboud, Y. Analysis of quantitative trait loci for chlorophyll fluorescence parameters reveal genetic differences in the regulation of PSII efficiency in maize seedlings.
and/or \( F_v' / F_m' \), these three parameters largely respond in parallel to changes in the environmental conditions (Baker and Oxborough, 2004). Changes in these fluorescence parameters are attributed to changes in the different fluorescence levels. For example, a decrease in \( F_o / F_m \), which describes the maximum quantum efficiency of PSII photochemistry, can be due to an increase in the minimal fluorescence of dark-adapted leaves \( (F_o) \), as occurs when PSII is damaged (Bradbury and Baker, 1986), or it can be caused by a decrease in the maximal fluorescence of dark-adapted leaves \( (F_m) \) (accompanied by a small decrease in \( F_o \)), which is due to sustainable down-regulation (Adams III et al., 2006). However, when analyzing the cause of changes in the \( F_o / F_m \) of different plants, the fluorescence levels per se are usually unsuitable for such an analysis, because additional factors may affect the general fluorescence level, in particular the amount of chlorophyll per leaf area. Similarly, additional information about changes in \( F_v / F_m \) and its component parts \( F_v' / F_m' \) and \( F_v / F_v' \) can be gained analyzing the fluorescence levels \( F_v', F_m', \) and \( F_o' \).

From a physiological point of view, the analysis of quantitative trait loci (QTLs) in a mapping population provides information about the interaction among different traits (Vreugdenhil et al., 2007). If the QTLs for different traits are at the same position on a genetic map, then pleiotropy is very likely, meaning that the QTLs are controlled by the same gene. This enables the identification of causal relationships among traits. Analyses of QTLs for photosynthesis have been conducted mostly by the means of chlorophyll fluorescence technique. For example, drought tolerance was studied based on the chlorophyll fluorescence in sunflower (Poormohammad Kiani et al., 2008), barley (Guo et al., 2008) and wheat (Yang et al., 2007). QTL studies of maize, based on the chlorophyll fluorescence, focused on the chilling tolerance of photosynthesis (Fracheboud et al., 2002; Fracheboud et al., 2004; Jompuk et al., 2005; Pimentel et al., 2005). In studies of a dent maize mapping population, the parental lines of which are characterized by contrasting chilling tolerance of photosynthesis, several QTLs for chlorophyll fluorescence parameters were detected under controlled (Fracheboud et al., 2004) and under field conditions (Jompuk et al., 2005). A comparison of these experiments revealed that the major QTLs for chilling tolerance of photosynthesis were stable across cold environments. However, it was not reported whether the expression of these major QTLs is caused by the temperature per se or by the excitation pressure. The expression of many cold-regulated genes is induced by an increase in the excitation pressure, as demonstrated for winter rye (Ndong et al., 2001). Therefore, the first goal of the present study was to examine the expression of the major QTLs for photosynthesis-related traits in dependence on temperature and light intensity. Second, the aim was to analyze the physiological causes of the genetic differences in the PSII operating efficiency in order to study the genetic basis of the major QTLs for photosynthesis. QTLs for several fluorescence parameters and fluorescence levels were determined for maize seedlings grown at suboptimal and optimal temperature at moderate light intensity as well as at optimal temperature at a higher light intensity. The results indicate that light intensity plays a major role in the expression of QTLs for chilling tolerance of photosynthesis. Furthermore, it was demonstrated that genetic differences in the PSII operating quantum efficiency can be affected independently by the PSII maximum efficiency and the \( Q_a \) redox state.

**Material and methods**

**Plant material and growth conditions**

A dent maize (\( Zea mays \) L.) mapping population in the \( F_{2:3} \) generation, which was derived from the chilling-tolerant line ETH-DH7 and the chilling-sensitive line ETH-DL3, was used for this QTL experiment (for details see Fracheboud et al., 2004). Seedlings were grown in climate chambers (PGW36, Conviron, Winnipeg, Canada) in 1-l pots containing a mixture of soil, peat and compost (Topf und Pikiererde 140, Rieder, Aarberg, Switzerland). Plants developed at optimal temperature (25/22 °C, day/night) at a medium light intensity of 400 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (25/ML) and at a high light intensity of 800 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (25/HL) as well as at suboptimal temperature (15/13 °C) under medium light intensity (15/ML). For plants grown at suboptimal temperature, the seedlings were first grown for six days at 25/22 °C. The photoperiod was 12 h and the humidity was 60/70% (day/night). The 226 families of the population as well as the parental lines were grown at intervals in blocks of six plants of 11 families.

**Chlorophyll fluorescence analysis**

Chlorophyll fluorescence parameters of light-adapted fully expanded third leaves were measured with an LI-6400 instrument equipped with an LI-6400-40 pulse-amplitude modulation fluorometer (LI-COR, Lincoln, NE, USA). The light intensity during measurements corresponded to the growth light intensity and was set at 400 or 800 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) with 10% blue (\( \lambda = 465 \) nm) and 90% red light (\( \lambda = 635 \) nm), and the temperature corresponded to the growth temperature, 15 °C or 25 °C. The fluorescence in light (\( F_v' \)) was recorded at a measuring light frequency of 0.25 kHz when photosynthetic CO2-fixation was stable, which usually took about 3 min. A saturation flash (0.8 s, >8000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and 20 kHz) was then applied to determine the maximum fluorescence...
cence in the light-adapted state \((F'_m)\). The actinic light was then turned off, and the leaf was illuminated by far red light \((\lambda = 740\text{ nm})\) at 1 kHz for 3 s to determine the ground fluorescence of light-adapted leaves \((F_0)\). The operating quantum efficiency of photosystem II (PSII) photochemistry \((F'_q/F'_m)\) was calculated as \((F'_m - F')/F'_m\). The operating PSII efficiency \((F'_q/F'_m)\) was \((F'_m - F'_o)/F'_m\), and the photochemical quenching factor \((F'_q/F'_m)\) was \((F'_m - F')/(F'_m - F'_o)\). The nomenclature of chlorophyll fluorescence parameters is according to Baker (2008).

The base fluorescence of dark-adapted leaves \((F_0)\) was determined after 30 to 60 min. in the dark at room temperature with a PAM-2000 fluorometer (Walz, Effeltrich, Germany) at a measuring light frequency of 600 Hz. The maximum fluorescence yield \((F_m)\) was then recorded during the application of a one-second saturation flash \((>8000\mu\text{mol m}^{-2}\text{s}^{-1})\) at a measuring light frequency of 20 kHz. The maximum quantum efficiency of PSII primary photochemistry \((F'_q/F'_m)\) was \((F'_m - F'_o)/F'_m\).

**Quantitative trait loci analysis**

The genetic map of the ETH-DL3 × ETH-DH7 population (Fracheboud et al., 2004) was slightly improved by adding three additional SSR markers. The QTL analysis was performed with QTL Cartographer v. 1.17b software (Basten et al., 1994). Joint analysis of the phenotypic data of the three growing environments by composite interval mapping was computed according to the JZmapqtl procedure of QTL Cartographer (Jiang and Zeng, 1995). For single-environment analysis, the Zmapqtl program module was employed for the composite interval mapping (Basten et al., 2005). In both cases, the genome was scanned at 2-cM intervals, and the window size was set at 30 cM. Cofactors were chosen using the forward-backward method of step-wise regression at \(p(F_m) = p(F_{out}) = 0.05\). The presence of a QTL was considered to be significant when the LOD score was higher than 5.5 for the joint analysis of the three environments. Additive effects of the detected QTLs were estimated by the Zmapqtl procedure of QTL Cartographer (Basten et al., 2005).

**Results**

Suboptimal growth temperature resulted in a marked reduction of the maximum quantum efficiency of PSII photochemistry \((F'_q/F'_m)\) (Table 1). Under these conditions, the chilling-tolerant line ETH-DH7 exhibited a considerably higher \(F'_q/F'_m\) than ETH-DL3. At optimal temperature, however, the difference between the genotypes was smaller, especially at high light intensity when the highest \(F'_q/F'_m\) values were observed. The situation was very similar for the genotypic difference in the PSII operating efficiency \((F'_q/F'_m)\), which was, however, significant only at suboptimal temperature. The genotypic difference in \(F'_q/F'_m\) was attributed mainly to a lower PSII maximum efficiency \((F'_q/F'_m)\) of ETH-DL3 than ETH-DH7. At suboptimal temperature \((15/ML)\) and at optimal temperature and high light intensity \((25/HL)\), when \(F'_q/F'_m\) was lower than at optimal temperature and moderate light intensity \((25/ML)\), the genotypic differences in \(F'_q/F'_m\) were highly significant. In contrast to the PSII efficiencies, the PSII efficiency factor \((F'_q/F'_o)\) was higher in the chilling-sensitive genotype ETH-DL3 than in ETH-DH7 when the seedlings developed at optimal temperature at moderate light intensity \((25/ML)\). With increasing excitation pressure, this difference became smaller and, at suboptimal growth temperature, ETH-DH7 was characterized by a higher \(F'_q/F'_o\) than ETH-DL3. In order to answer the question as to whether the genotypic differences in the PSII efficiency and in the PSII efficiency factor are monogenic or caused by the interaction of various genetic regions, a QTL analysis was conducted using an \(F_2:3\) mapping population derived from the chilling-sensitive genotype ETH-DL3 and the chilling-tolerant ETH-DH7. The phenotypic analysis of the \(F_2:3\) population revealed a high variation of \(F'_q/F'_m\), \(F'_q/F'_o\) and \(F'_q/F'_o\) in seedlings grown at suboptimal temperature but low variation of these traits in seedlings grown at optimal temperature (Figure 1). Furthermore, a transgressive segregation was found in both directions. Within and across growth conditions, \(F'_q/F'_o\) and \(F'_q/F'_o\) showed a close linear correlation, while \(F'_q/F'_o\) was clearly less correlated with \(F'_q/F'_m\).

**Table 1: Overview of statistics for the maximum quantum efficiency of PSII photochemistry \((F'_q/F'_m)\), PSII operating efficiency \((F'_q/F'_m)\), PSII maximum efficiency \((F'_q/F'_m)\) and PSII efficiency factor \((F'_q/F'_o)\) of the parental lines grown at 15 °C and 400 µmol m\(^{-2}\) s\(^{-1}\) (15/ML), at 25 °C and 400 µmol m\(^{-2}\) s\(^{-1}\) (25/ML) and at 25 °C and 800 µmol m\(^{-2}\) s\(^{-1}\) (25/HL). Values are means ± SD of at least 11 replicates.**

<table>
<thead>
<tr>
<th>Trait</th>
<th>Genotype</th>
<th>Environment</th>
</tr>
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<tbody>
<tr>
<td>(F'_q/F'_m)</td>
<td>ETH-DH7</td>
<td>0.723 ± 0.018</td>
</tr>
<tr>
<td></td>
<td>ETH-DL3</td>
<td>0.493 ± 0.085</td>
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<tr>
<td>(F'_q/F'_o)</td>
<td>ETH-DH7</td>
<td>0.181 ± 0.049</td>
</tr>
<tr>
<td></td>
<td>ETH-DL3</td>
<td>0.067 ± 0.042</td>
</tr>
<tr>
<td></td>
<td>***</td>
<td>ns</td>
</tr>
<tr>
<td>(F'_q/F'_o)</td>
<td>ETH-DH7</td>
<td>0.258 ± 0.062</td>
</tr>
<tr>
<td></td>
<td>ETH-DL3</td>
<td>0.108 ± 0.052</td>
</tr>
<tr>
<td></td>
<td>***</td>
<td>ns</td>
</tr>
<tr>
<td>(F'_q/F'_o)</td>
<td>ETH-DH7</td>
<td>0.699 ± 0.100</td>
</tr>
<tr>
<td></td>
<td>ETH-DL3</td>
<td>0.601 ± 0.095</td>
</tr>
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<td>***</td>
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Comparison of mean values at: ***: \(P < 0.001\); **: \(P < 0.01\); *: \(P < 0.05\); (*) \(P < 0.1\); ns, not significant.
The quantitative trait locus (QTL) analysis under three sets of growth conditions (joint analysis) revealed three genomic regions, which were associated with the phenotypic variation of at least three of the four analyzed traits (Table 2). These genomic regions were located on chromosome 1 (216-230 cM), chromosome 3 (73-93 cM) and chromosome 6 (224-248 cM). The latter seems to contain two independent QTLs, one at about 226 cM and a second at 248 cM. Four additional QTLs were detected for $F_v/F_m$, located on chromosomes 2 (123 cM), 4 (137 cM), 5 (111 cM) and 6 (91 cM).

![Figure 1: Relationship of the PSII operating efficiency ($F_q'/F_m'$), PSII maximum efficiency ($F_v'/F_m'$) and the PSII efficiency factor ($F_q'/F_v'$) in the third leaf of maize grown at 15 °C and 400 µmol m$^{-2}$ s$^{-1}$ (15/ML), at 25 °C and 400 µmol m$^{-2}$ s$^{-1}$ (25/ML); white circles: F$_2$ families grown at 25 °C and 400 µmol m$^{-2}$ s$^{-1}$; black circles: F$_2$ families grown at 15 °C and 400 µmol m$^{-2}$ s$^{-1}$ (25/ML); white triangles: F$_2$ families grown at 25 °C and 800 µmol m$^{-2}$ s$^{-1}$; gray diamonds: tolerant parent ETH-DH7; gray squares: sensitive parent ETH-DL3. Values represent averages of six to nine replicates.]

![Table 2: Main characteristics of QTLs for the maximum quantum efficiency of PSII photochemistry ($F_v/F_m$), PSII operating efficiency ($F_q'/F_m'$), PSII maximum efficiency ($F_v'/F_m'$) and the PSII efficiency factor ($F_q'/F_v'$) of maize seedlings grown at 15 °C and 400 µmol m$^{-2}$ s$^{-1}$ (15/ML), at 25 °C and 400 µmol m$^{-2}$ s$^{-1}$ (25/ML) and at 25 °C and 800 µmol m$^{-2}$ s$^{-1}$ (25/HL) with an LOD score above a threshold of 5.5 for the joint analysis.]

The quantitative trait locus (QTL) analysis under three sets of growth conditions (joint analysis) revealed three genomic regions, which were associated with the phenotypic variation of at least three of the four analyzed traits (Table 2). These genomic regions were located on chromosome 1 (216-230 cM), chromosome 3 (73-93 cM) and chromosome 6 (224-248 cM). The latter seems to contain two independent QTLs, one at about 226 cM and a second at 248 cM. Four additional QTLs were detected for $F_v/F_m$, located on chromosomes 2 (123 cM), 4 (137 cM), 5 (111 cM) and 6 (91 cM).
The QTL on chromosome 1 was expressed under suboptimal growth temperature but not at optimal temperature (Table 2). While this QTL was significant for \( F_v/F_m \), \( F_o/F_m \) and \( F_m \), it was not found for \( F_v'/F_m' \). In contrast to the QTL on chromosome 1, the QTL on chromosome 3 was particularly important for the expression of the traits at optimal temperature but not at suboptimal temperature. For \( F_v/F_m \), this QTL was significant only at low excitation pressure (ML/25). The two QTLs in the telomeric region of chromosome 6 were significant for the PSII efficiencies (\( F_v/F_m \), \( F_q'/F_m' \) and \( F_v'/F_m' \)) under all growth conditions, with the exception of \( F_v/F_m \) at 25/ML. The latter resulted in a high QTL-environment interaction of this QTL for \( F_v/F_m \). In particular, a very high LOD score was found for these three traits at high light intensity and, additionally, for \( F_v/F_m \) in seedlings grown at suboptimal temperature. The phenotypic variation of the PSII efficiency factor, \( F_q'/F_v' \), was not associated with this genomic region.

Figure 2: Additive effects on chromosome 1 for the maximum quantum efficiency of PSII photochemistry (\( F_v/F_m \)) and its compounds \( F_o, F_m \) and \( F_v \) (A-C), PSII operating efficiency (\( F_o/F_m' \)), PSII maximum efficiency (\( F_v/F_m' \)) and PSII efficiency factor (\( F_q'/F_v' \)) (D-F) and its compounds \( F_q', F_m', F_o' \) and \( F_v' \) (G-I) in the third leaf of maize grown at 15 °C and 400 µmol m\(^{-2}\) s\(^{-1}\) (15/ML; A, D and G), at 25 °C and 400 µmol m\(^{-2}\) s\(^{-1}\) (25/ML; B, E and H) and at 25 °C and 800 µmol m\(^{-2}\) s\(^{-1}\) (25/HL; C, F and I). Positive additivity indicates that high values of the trait were inherited from the tolerant parent (ETH-DH7), and negative additivity indicates that high values of the trait were inherited from the sensitive parent (ETH-DL3). For each trait, regions with an LOD score above 2.5 are indicated by bold lines.
After finding the major genomic regions, which are associated with the expression of the studied traits, a detailed analysis of the additive effects of these QTLs was conducted by single-environment analysis. Moreover, the basic fluorescence parameters $F_o$, $F_m$, $F_v$, $F'_o$, $F'_m$, $F'_v$ were included in this analysis to obtain further information about the nature of the changes in the PSII efficiency and the PSII efficiency factor. The focus was on the additivity of the QTL, because it describes to which extent the allele at the QTL affects the trait. Here, a positive additivity indicates that high values of the trait were inherited from the chilling-tolerant parent (ETH-DH7); negative additivity implies that the allele in the QTL region of the chilling-sensitive parent (ETH-DL3) is responsible for the high trait values.

The QTL for $F_v/F_m$ on chromosome 1 of seedlings grown at suboptimal temperature (15/ML) was characterized by positive additivity (Figure 2). The high values for $F_v/F_m$ in the $F_{2:3}$ families, which carried the ETH-DH7 allele in this position, were mainly due to an increase in the variable fluorescence ($F_v$), which seemed to be caused by a small reduction in the minimal fluorescence ($F_o$) and an increase in the maximal fluorescence ($F_m$). A similar situation was found in seedlings grown at optimal temperature at moderate light intensity (25/ML) but additive effects were considerably weaker (Figure 2B). The single-environment analysis showed that this chromosomal region was involved in the phenotypic variation of $F_v'/F_m'$ at suboptimal temperature (LOD score of 3.3). This analysis also revealed that high values of

**Figure 3:** Additive effects on chromosome 3 for chlorophyll fluorescence parameters. For details see Figure 2.
$F_{q'}/F_{m'}$, which were inherited from the chilling-tolerant parent, were probably caused by both high $F_{v'}/F_{m'}$ and high $F_{q'}/F_{v'}$ (Figure 2C). The analysis of the additivity for the different fluorescence levels indicated that the allele of ETH-DH7 was responsible for an increase in the fluorescence level ($F_{q'}/F_{m'}$ and $F_{o'}$) as well as in the variable fluorescence ($F_{v'}$) and the photochemical quenching of fluorescence by open PSII centers ($F_{q'}$) (Figure 2F). The general increase in the fluorescence level may be caused by a QTL for leaf greenness in this position that is characterized by a positive additivity (data not shown).

The QTL on chromosome 3 was characterized by expression only at optimal temperature, especially at moderate light intensity, as well as by negative additivity, indicating that high trait values were inherited from the chilling-sensitive genotype ETH-DL3 (Figure 3). The analysis of the additivity of the QTL revealed that the increase in $F_{q'}/F_{m'}$, due to the ETH-DL3 allele, was probably caused by an increase of $F_{q'}/F_{v'}$ (Figure 3E), which in turn was due mainly to a higher $F_{q'}$ (Figure 3H). At high light intensity, a similar association was found between $F_{q'}/F_{m'}$ and $F_{q'}/F_{v'}$, but with weaker additive effects and with no distinct changes in the fluorescence. The change in $F_{q'}/F_{m'}$ seemed to have an effect on $F_{q'}/F_{m'}$ only at optimal temperature and moderate light intensity (25/ML).

The QTL analysis of the population grown at subop-

**Figure 4:** Additive effects on chromosome 6 for chlorophyll fluorescence parameters. For details see Figure 2.
timal temperature revealed a QTL for $F_v'/F_m'$ and $F_o'/F_m'$ on chromosome 3 with positive additivity (Figure 3 D). This QTL, however, seems to be distinct from the former QTL, since the LOD score peaked at 55 cM for the 15/ML-plants and at 102 cM for the 25/ML-plants (data not shown). Moreover, the QTL analysis of plants grown under high light intensity (25/HL) revealed two distinct LOD peaks in the corresponding positions.

The two QTLs for $F_o/F_m$, $F_o'/F_m'$ and $F_v/F_m'$ on the telomeric region of chromosome 6 showed positive additivity, revealing that the allele of ETH-DH7 was responsible for the high values of these parameters (Figure 4). The additive effects were slightly stronger for the QTL at ~226 cM compared to the QTL at 248 cM. The extent of the additivity for $F_o'/F_m'$ and $F_v'/F_m'$ was similar under all growth conditions. In contrast, the effect on $F_o/F_m$ increased considerably with increasing excitation pressure. Under these conditions (15/ML and 25/HL), the high $F_o/F_m$ values due to the allele from ETH-DH7 were due mainly to lower values of $F_o$. At low excitation pressure (25/ML), the ETH-DH7 allele caused a small but significant reduction in $F_o$, $F_m$ and $F_v$ but had no effect on $F_o/F_m$ (Figure 4B). At optimal temperature (25/ML and 25/HL), the increase in $F_o'/F_m'$ due to the ETH-DH7 allele was associated with an increase in $F_v'/F_m'$, which was attributed to a lower $F_o'$ and $F_v'$. In contrast, the positive additivity of these QTLs for $F_o'/F_m'$ and $F_v'/F_m'$ was associated with positive additivity for $F_o'$ and $F_v'$.

Discussion

Since the PSII operating efficiency ($F_o'/F_m'$) is the product of the PSII maximum efficiency ($F_v'/F_m'$) and the PSII efficiency factor ($F_v'/F_o'$), changes in $F_v'/F_m'$ can be caused by changes in $F_v/F_m$ and in $F_v/F_o'$. Compared to seedlings grown at optimal temperature and moderate light intensity (25/ML), the reduction of $F_v'/F_m'$ in seedlings grown and measured at suboptimal temperature (15/ML) as well as in seedlings that grew and were measured at high light intensity (25/HL) was due to a reduction of both $F_v'/F_m'$ and $F_o'/F_v'$. By comparing the two genotypes, it is obvious that the ratio of $F_v'/F_m'$ to $F_o'/F_o'$ is not fixed at a certain value for $F_o'/F_m'$. At optimal temperature, the chilling-sensitive genotype ETH-DL3 was characterized by a lower $F_v'/F_m'$ but a higher $F_o'/F_o'$ than the chilling-tolerant ETH-DH7. The lower $F_v'/F_m'$ indicates a larger amount of non-photochemical quenching. The higher $F_o'/F_o'$ of ETH-DL3 suggests a higher proportion of closed PSII reaction centers under these conditions. Since the relationship between $F_v'/F_m'$ and the $Q_o$ redox state is curvilinear when there is connectivity between the PSII reaction centers (Joliot and Joliot, 1964), $F_v'/F_m'$ reflects the redox state of $Q_o$ only in the ‘puddle’ antenna model, which should not be applied to interpret chlorophyll fluorescence data of higher plants. Light response curves of $q_L$, which estimates the fraction of open PSII centers in the open state based on the ‘lake’ antenna model, which considers connectivity between PSII reaction centers (Kramer et al., 2004b), revealed a higher $q_L$ of ETH-DL3 than of ETH-DH7 (data not shown). Even at suboptimal temperature, the $q_L$ of ETH-DL3 was higher than that of ETH-DH7 when measured at low light intensity (data not shown). Since the curvilinearity of the relationship between $F_o'/F_v'$ and the redox state of $Q_o$ decreases with increasing level of non-photochemical quenching (Baker et al., 2001), the above-mentioned findings strongly indicate that, under low excitation pressure, the $Q_o$ of ETH-DL3 was oxidized to a greater extent than that of ETH-DH7. However, this does not seem to be the case when plants grew in the field (Jompuk et al., 2005); both genotypes exhibited similar values of $F_o'/F_v'$. The QTL experiment revealed that the genotypic differences in the PSI efficiency and the PSII efficiency factor between the studied genotypes are caused by several genes, respectively genomic regions, which were also found in earlier studies to be involved in the expression of photosynthesis-related traits in this mapping population (Fracheboud et al., 2004; Jompuk et al., 2005). The comparison of QTL expression under different growth conditions showed that, among the major QTLs, which were identified at suboptimal temperature (15/ML), the QTL for photosynthesis-related traits on chromosome 1 seemed to be regulated by the temperature but slightly by the excitation pressure. On the other hand, the QTL on chromosome 6 was also found for $F_o/F_m$ at high light intensity (25/HL), supporting the conclusions drawn from the field experiment of Jompuk et al. (2005) that the expression of this QTL tends to be regulated by the excitation pressure rather than by the low temperature per se. The same seems to be true for the QTL on chromosome 2 (data not shown), which was found to be involved in PSI maximum efficiency of light-adapted leaves ($F_o'/F_m'$) (Fracheboud et al., 2004).

Co-localization of QTLs for different traits indicates a pleiotropic effect, which means that the traits are controlled by the same gene. Consequently, the QTL analysis is a very powerful tool for studying the interaction among traits. Based on this approach, it was revealed that genotypic differences in $F_o'/F_m'$ were due to genotypic differences in $F_v'/F_m'$, as for the QTL on chromosome 6, or due to genotypic differences in $F_o'/F_v'$, as for the QTL on chromosome 3, or due to both $F_v'/F_m'$ and $F_o'/F_v'$, as for the QTL on chromosome 1. Consequently, genes seem to exist, which can influence the $Q_o$ redox state without having a strong genotypic effect on non-photochemical quenching, as shown for the QTL on chromosome 3. On the other hand, there seem to be genes, which
modulate photosynthesis upstream of PSII by targeting directly or indirectly non-photochemical quenching mechanisms without inducing genotypic differences in the QA redox state, such as the gene(s) underlying the QTL on chromosome 6. A closer look at the QTL on chromosome 3 revealed that the increase in $F_{0}'/F_{m}'$ and $F_{a}'/F_{m}'$, due to the allele of ETH-DL3, was associated with a significantly higher $F_a'$, reflecting a greater amount of photochemical quenching of fluorescence by open PSI centers, without having a statistically significant effect on $F_{v}'/F_{m}'$. Based on these characteristics, it is assumed that the underlying gene or genes affect photosynthesis downstream of PSII ($P_{680}$). The $spS2$ gene, which codes sucrose phosphate synthase, has been previously discussed as candidate gene for this QTL (Jompuk et al., 2005). Based on the present results, another potential candidate gene, $psaN$, was identified, which is located closer to the QTL peak. This gene codes the PSI-N subunit of photosystem I. The PSI-N subunit is located on the luminal side of PSI and has been suggested to be involved in the docking of plastocyanin to PSI (Jensen et al., 2003). However, a recent study indicates that a direct interaction of PSI-N with plastocyanin is unlikely (Amunts et al., 2007). Whatever the precise function of PSI-N, Arabidopsis mutants lacking PSI-N showed a very similar phenotype to $F_{2:3}$ plants carrying the ETH-DH7 allele in the QTL position on chromosome 3; the mutants were characterized by a reduction of $F_a/F_{m}'$, which was due to a decrease in $F_a'/F_{m}'$, while non-photochemical quenching (determined as $q_a$) was not affected (Haldrup et al., 1999). A shift in the ratio between linear electron transport and cyclic electron transport around PSI could explain why a higher $F_a'/F_{m}'$ and $F_a'/F_{m}'$ can be maintained in plants carrying the ETH-DL3 allele on chromosome 3 without having an effect on $F_a/F_{m}'$. This, however, assumes that $F_a'/F_{m}'$ similarly responds to the $\Delta$pH in both genotypes, which may not necessarily be the case, because the sensitivity of the non-photochemical quenching to the luminal pH can be altered (Kramer et al., 2004a).

In-depth analysis of the QTL in the telomeric region of chromosome 6 revealed that $F_a/F_{m}'$ was solely modulated by $F_a'/F_{m}'$ at optimal temperature only, while genotypic alterations in the QA redox state were associated with the shift in the PSII operating efficiency at suboptimal temperature. Moreover, the increase in $F_a/F_{m}'$ at optimal temperature due to the ETH-DH7 allele in this QTL position was accompanied by a reduction of the fluorescence intensity, especially of the minimal fluorescence in light- ($F_{a}'$) and dark-adapted leaves ($F_a$) but also to some extent of the maximal fluorescence. The intensity of the fluorescence signal can depend on the amount of chlorophyll. However, since leaf greenness, i.e. the content of chlorophyll, is not affected by this QTL (Fracheboud et al., 2004; Jompuk et al., 2005), the genotypic differences in the overall fluorescence level may be attributed to constitutive structural differences in PSII, which in turn influence the photosynthetic efficiency of leaves exposed to light. At suboptimal temperature, this structural alteration of the PSII may be the cause of the greater requirement for photoprotection and down-regulation in plants carrying the ETH-DL3 allele in this QTL position, as indicated by the lower $F_a/F_{m}'$ values in conjunction with a lower $F_{v}'/F_{m}'$. The fraction of the reduction of $F_v/F_{m}$ in these plants, which was due to a decrease in $F_{m}'$, appears to be caused by persistent $\Delta$H-independent down-regulation, probably by sustained phosphorylation of the PSII core protein D1 (Adams III et al., 2006). The higher minimal fluorescence ($F_o$) in these plants, however, suggests damage or loss of PSII reaction centers as a cause of the lower $F_v/F_{m}$ (Baker, 2008). An accumulation of damaged PSII reaction centers can be caused either by an increase in the rate of photodamage or by a decrease in the rate of D1 protein repair (Melis, 1999). Our first impression is that the genotypic differences in $F_v/F_{m}$, which became more pronounced with increasing excitation pressure, suggest an allelic effect on the repair of the D1 protein; due to the identical light conditions the rate of photodamage should be the same, according to the photodamage model, in which PSII is considered to be a 'photon counter' (Park et al., 1996). Genotypic differences in the repair of the D1 protein could be explained by the presence of the EST PC0153568 in the QTL region, which is very similar to the gene of the serine protease DegP1 from Arabidopsis. It is well documented that DegP1 plays an important role in the repair of damaged D1 protein of the PSII reaction center (Kapri-Pardes et al., 2007) and, consequently, it could be involved in the observed phenotype.

However, there is also evidence that the genotypic difference in the damage of the PSII reaction center is caused by different rates of photodamage. It is known that the size of PSII antenna has an effect on the rate of photodamage (Melis, 1999). Although the QTL in the telomeric region of the long arm of chromosome 6 is not associated with the chlorophyll content, it showed a strong QTL for the chlorophyll $a/b$ ratio in seedlings grown at suboptimal temperature (Leipner and Mayer, 2009); the allele of ETH-DH7 was responsible for the higher chlorophyll $a/b$ ratio. Since the light-harvesting complexes contain chlorophyll $a$ and $b$, while the reaction centre core complex contains only chlorophyll $a$ (Peter and Thornber, 1991), the ETH-DH7 allele may result in a lower ratio of the light-harvesting complex to the core complex. This would result in a lower excitation pressure on the PSII reaction center and, subsequently, to a lower rate of photodamage. A potential candidate gene, located very close to the peak of this QTL and
which could explain the observed phenotype, is \textit{cab-m7} coding for the LHCII protein \textit{Lhcbm7}. \textit{Cab-m7} is preferentially expressed in maize mesophyll cells and is strongly induced upon illumination (Becker et al., 1992). Co-localization of \textit{Lhcb} genes with QTLs for cold-induced photoinhibition was also reported in another study of maize QTLs (Pimentel et al., 2005). Whatever the precise molecular cause of the QTL on chromosome 6, structural changes in PSII upstream of P$_{680}$ induce changes in the PSII efficiency, which lead to flexible and sustained down-regulation as well as to damage of the PSII reaction center when seedlings develop at suboptimal temperature.

In summary, the results revealed that $F_{q'}/F_{m'}$ can be modulated independently by $F_{v'}/F_{m'}$ and $F_{q'}/F_{v'}$. In particular, the data showed that, under high excitation pressure, the PSII operating efficiency is mainly controlled by its maximal efficiency, while components downstream of P$_{680}$ additionally modulate the PSII operating efficiency under low excitation pressure. Moreover, genotypic differences in the maximum quantum efficiency of PSII photochemistry, caused by growth at suboptimal temperature, were attributed both to persistent down-regulation of PSII and damage to the PSII reaction center. Although the presented data provide information about the causes of genotypic differences in the response of the photosynthetic machinery to environmental conditions, they also give valuable insight into the overall response of the photosynthetic apparatus to environmental factors.
Chapter 8

Genetic analysis of chilling-acclimation at different temperatures at night in maize seedlings*

Suboptimal temperature during maize (Zea mays L.) seedlings growth results in a decrease of the photosynthetic efficiency due to a combination of temperature and light stress. Details remain scant on the impact of (low) night temperatures on the photosynthetic activity. For a better understanding of the role of the temperature at night on the acclimation of the photosynthetic apparatus to suboptimal temperature, a QTL experiment was conducted with the B73 × Mo17 population (IBM302 population). Seedlings were grown under optimal temperature (24/22 °C) or under suboptimal temperature (17 °C) during the day and at 13 or 6 °C during the night. The two parental lines, B73 and Mo17, grown at suboptimal temperature during the day responded differently to the temperature at night, as revealed by measurements of the operating quantum efficiency of PSII ($F_{q'}/F_{m'}$), the maximum quantum efficiency of PSII primary photochemistry ($F_{v'}/F_{m}$) and leaf greenness (SPAD). While Mo17 showed very little response to the temperature at night, B73 exhibited a lower photosynthetic performance at 13 °C than at 6 °C at night. At 17/6 °C the photosynthetic efficiency of both genotypes was similar. These observations were supported by the QTL analysis. The major QTL for photosynthesis-related traits, which was detected on chromosome 5 with a favorable allele inherited from Mo17, had a lower additivity at temperature of 6 °C than at 13 °C during the night. As potential candidate genes for this locus, ivr2 (coding for an acid vacuolar invertase) and a2 (coding for an anthocyanidin synthase) were identified. However, QTL analysis for invertase activity and anthocyanin content did not confirm either of them being involved in the expression of the major QTL for chilling tolerance of photosynthesis. Instead, QTLs were detected for invertase activity nearby the ivr1 gene and for anthocyanin content close to the r1 locus, which is an important locus in the regulation of the anthocyanin synthesis. Comparative QTL analyses of photosynthetic traits of this population compared to analyses reported in other studies revealed conserved QTLs on chromosome 6 and 8.

Introduction

Studies of plant acclimation to extreme environments are of outstanding importance in agriculture, especially for important crops like maize (Zea mays L.). Maize, a C₄ plant, is considered to have originated from a single domestication in southern Mexico around 9,000 years ago. The oldest surviving maize types are those of the Mexican highlands (Matsuoka et al., 2002). Temperature is one of the most important factors determining maize cultivation in temperate regions. Nevertheless, cultivation of maize took place in early civilization in North America and later in Europe (Rebourg et al., 2003). In temperate regions, maize is cultivated between spring and autumn and is, consequently, mostly affected by low temperature during germination, heterotrophic growth, early autotrophic growth and later during the grain filling period (Lee et al., 2002a; Ying et al., 2002). In North America and Europe, the optimal temperature for growth, which is between 20 and 30 °C, is rarely reached. Sudden drops in temperature, which frequently occur during the early seedling development, have a negative impact on the physiology of maize (Foyer et al., 2002). Furthermore, suboptimal temperature retards germination and the growth of maize seedlings. Chlorotic leaves are the most visible indications of malfunction of the chloroplasts in seedlings that developed at suboptimal temperature (Haldimann et al., 1996; Nie and Baker, 1991; Nie et al., 1995). This is accompanied by inhibition of photosynthesis due to perturbations of the photosynthetic electron transport (Haldimann et al., 1996), the carbon reduction cycle (Kingston-Smith et al., 1997) and the control of stomatal conductance (Allen and Ort, 2001). These types of chilling-induced damage to the photosynthetic apparatus have been described as being useful markers for estimating the chilling tolerance of maize seedlings (Fracheboud et al., 1999).

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Guerra-Peraza, O., Leipner, J., Reimer, R., Nguyen, H.T., Stamp P., Fracheboud Y. Night temperature affects the genetic control of cold-acclimation in maize seedlings.
The central role of photosynthesis within the context of chilling stress is based on the fact that low temperatures induce a low intensity stress. Because of the cold-induced reduction of the activity of the carbon cycle, only a small part of the absorbed light energy can be used to drive this process. Therefore, it is not surprising that the expression of many cold-regulated genes is induced by this increase of the excitation pressure (Ndong et al., 2001). In order to safely dissipate the excess excitation, plants have developed mechanisms to down-regulate photosystem II, in which the xanthophyll cycle pigments play an important role (Adams III et al., 2006). In maize seedlings, growth at suboptimal temperature results in the accumulation of xanthophyll-cycle pigments in the leaves, especially when they develop at a higher light intensity (Haldimann et al., 1995). Such chilling-acclimated leaves are characterized by a lower photosynthetic efficiency; they are, however, better able to withstand photoinhibition and photooxidative stress than leaves that developed under more favorable conditions (Haldimann et al., 1996; Leipner et al., 1997). Other important factors that are involved in the acclimation of maize seedlings to the chilling-induced light stress are the accumulation of antioxidants and scavenging enzymes (Kingston-Smith et al., 1999; Kocsy et al., 1996; Leipner et al., 1997) and of anthocyanin (Pietrini et al., 2002). However, temperature per se seems to have an effect as well. In particular, changes in the lipid composition of membranes are modulated by the temperature (Nishida and Murata, 1996) and seem to be associated with the chilling tolerance of maize seedlings (Kaniuga et al., 1999). Although it is well known that most of these acclimation mechanisms are controlled by both temperature and light intensity, little is known to which extent temperature at night affects the acclimation of maize to chilling stress. There are indications that reduced temperature at night aggravates the cold-induced damage (Saropulos and Drennan, 2002; Ying et al., 2000) and strongly affects leaf growth by increasing cell cycle time and decreasing cell production (Rymen et al., 2000). The extent of these alterations seem to be genotype dependent, as it was demonstrated in grapevine (Bertamini et al., 2007). On the other side, acclimation at low temperature during night can increase the chilling-tolerance of photosynthesis and seems to be even beneficial for photosynthesis at high temperature (Pittermann and Sage, 2001). The analysis of quantitative trait loci (QTL) is a useful tool for locating the genomic regions responsible for the expression of traits of interest like chilling tolerance. Furthermore, it enables us to unravel the interaction of complex traits. A number of QTL studies focused on the chilling tolerance of maize seedlings (Fracheboud et al., 2002; Fracheboud et al., 2004; Hund et al., 2005; Jompuk et al., 2005; Pimentel et al., 2005; Presterl et al., 2007). However, the effects of temperature at night on the acclimation of maize seedlings to chilling have not been subjected to a QTL analysis. Consequently, this study aimed for (i) determining chromosomal regions that are important in the adaptation of maize to chilling stress and (ii) determining the possible influence of temperature at night on the cold-acclimation. To achieve these goals, the IBM302 maize population (Lee et al., 2002b; Sharopova et al., 2002), which segregated for photosynthetic traits when exposed to two different low temperatures at night under controlled growth conditions, was studied for the presence of QTLs in seedlings grown at optimal temperature and seedlings grown at suboptimal temperature with two different chilling treatments during the dark phase.

Materials and methods

Plant material and culture conditions

The two parental lines, B73 and Mo17, and 295 of their 302 intermated recombinant inbred (RI) lines, known as the IBM302 population (Lee et al., 2002b; Sharopova et al., 2002), were kindly provided by the Maize Genetics Cooperation Stock Center (http://maizecoop.crops.uiuc.edu). The seeds were first multiplied by selfing the RI lines in the field in Switzerland during summer 2003. The resulting seeds were used in the experiments described here. The seedlings were grown in two growth chambers (PGW36, Conviron, Winnipeg, Canada) in pots (10 × 10 × 10 cm) containing a commercial mixture of soil, peat and compost (Topf und Pikiererde 140, Ricoter, Aarberg, Switzerland). Three seeds were sown in each pot and thinned to one plant per pot after eight days. The plants were grown until the third leaf was fully expanded. The control plants grew for 14 days at 24/22 °C (day/night) at a photoperiod of 12 h at 400 µmol quanta m⁻² s⁻¹ and a relative humidity of 60/70% (day/night). Chilling-treated plants were first grown for eight days as control plants and then for 20 days at 17/13 °C (day/night) or 24 days at 17/6 °C (day/night); the other conditions were the same as for the control plants. One run consisted of one plant of each of the RI lines and six to eight plants of each parental line in the same growth chamber. For each of the three temperature regimes, there were two runs, one in each of the two growth chambers. For the verification of candidate genes, the 295 RI lines of the IBM302 population were grown as described above at 24/22 °C and 17/13 °C (day/night).

Measurements of traits

The chlorophyll fluorescence parameters were measured on the third fully expanded leaf with a PAM-2000 fluorometer (Walz, Effeltrich, Germany).
equipped with a leaf-clip holder (2030-B). The PSII operating efficiency \( \left( \frac{F_v}{F_m} \right) \) was measured under growth conditions according to Genty et al. (1989) with the frequency of the measuring beam set at 20 kHz. The \( \frac{F_v}{F_m} \) was calculated as \( \left( \frac{F_m - F_v}{F_m} \right) \). The minimal fluorescence of dark-adapted leaves \( (F_o) \) was determined after 30 to 60 min in the dark at a measuring light frequency of 600 Hz. The maximal fluorescence \( (F_m) \) was then recorded during a one-second saturation flash (\( > 8000 \mu \text{mol quanta m}^{-2} \text{s}^{-1} \)) at a measuring light frequency of 20 kHz. The maximum quantum efficiency of PSII photochemistry \( (F_v/F_m) \) was calculated empirically by 1,000 permutations with a significance level of \( \alpha = 0.05 \) (Churchill and Doerge, 1994). The results of the composite interval mapping were verified by bootstrap analysis, conducting 200 bootstrap re-samplings with the QTL Cartographer procedure of QTL Cartographer 1.17, as well as by joint analysis of both repetitions using the \texttt{Jzmapqtl} procedure of QTL Cartographer 1.17.

Results

Effects of growth temperature

To better understand how the temperature at night influences the photosynthetic activity of maize seedlings, seedlings were examined at two low-temperature regimes. Seedlings grown at 17/13 °C or 17/6 °C were characterized by a lower PSII operating efficiency \( (F_v/F_m) \) and a lower maximum quantum efficiency of PSII photochemistry \( (F_v/F_m) \), paler leaves (SPAD) and a slower growth rate than seedlings, seedlings were examined at two low-temperature regimes. Seedlings grown at 17/13 °C or 17/6 °C were characterized by a lower PSII operating efficiency \( (F_v/F_m) \) and a lower maximum quantum efficiency of PSII photochemistry \( (F_v/F_m) \), paler leaves (SPAD) and a slower growth rate than seedlings that developed at 24/22 °C (Figure 1). The decrease in \( F_v/F_m \) was due to an increase in the ‘dark’ level of the chlorophyll fluorescence \( (F_o) \) in seedlings...
grown at suboptimal temperature (17/13 °C or 17/6 °C) and, additionally, due to a decrease in the maximal fluorescence \( (F_m) \) in seedlings exposed to 6 °C at night (Figure 1).

In leaves developed at 24/22 °C, the values of the studied traits of both parental lines were similar. However, B73 was characterized by a significantly higher \( F_v/F_m \), caused by a lower \( F_o \), compared to Mo17. The traits differed markedly when seedlings were grown at suboptimal temperature. The ANOVA analysis of the effect of the temperature at night on the two parents grown at suboptimal temperature indicated that the \( F_q'/F_m' \), \( F_v/F_m \) and SPAD of the two parental lines responded differently (Table 1). For \( F_v/F_m \) and SPAD, B73 showed significantly higher values than Mo17 when the seedlings were grown at 17/6 °C. The PSII operating efficiency \( (F_q'/F_m') \) was also higher in B73 than in Mo17 at 17/6 °C, but was significantly lower when seedlings were grown at 17/13 °C. Similarly, \( F_v/F_m \) was higher for Mo17 compared to B73 at 17/6 °C. This suggests that the decrease in the photosynthetic performance, caused by the suboptimal growth temperature, was minimized in B73, but not in Mo17, due to the low temperature at night.

In the RI population, lowering the temperature at night from 13 °C to 6 °C significantly decreased the medians of \( F_v/F_m \), \( F_m \) as well as the growth rate of the plants; it increased leaf greenness but had no influence on \( F_q'/F_m' \) and \( F_o \) (Figure 1). The result was the same for the means of the traits, as revealed by \( t \)-tests (data not shown). All traits showed moderate values (around 0.5) for the broad sense heritability \( (h^2) \), with values somewhat lower for \( F_v/F_m \) \( (h^2 = 0.392) \) and higher for SPAD \( (h^2 = 0.623) \); the correlation between the replications was significant \( (p < 0.1) \) for all traits.

### Quantitative trait loci analysis

To investigate the genetic basis of the effect of temperature at night on cold acclimation, QTL mapping

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**Table 1:** ANOVA statistics of chlorophyll fluorescence parameters, leaf greenness (SPAD) and growth rate (GR) in the parental lines grown at 17/13 °C and 17/6 °C. *, **, *** indicate a significant effect at \( P < 0.05, 0.01 \) and 0.001, respectively; NS: no significant effect.

<table>
<thead>
<tr>
<th>Factor</th>
<th>( F_q'/F_m' )</th>
<th>( F_v/F_m )</th>
<th>( F_o )</th>
<th>( F_m )</th>
<th>SPAD</th>
<th>GR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>**</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Temperature</td>
<td>***</td>
<td>NS</td>
<td>***</td>
<td>**</td>
<td>NS</td>
<td>***</td>
</tr>
<tr>
<td>Geno. × Temp.</td>
<td>***</td>
<td>***</td>
<td>NS</td>
<td>NS</td>
<td>*</td>
<td>NS</td>
</tr>
</tbody>
</table>

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**Figure 1:** Box-whisker plots for traits distribution. The horizontal lines near the center of the boxes indicate the median values of the traits; the bottom and top sections of the boxes represent the values of the first and third quartiles. The whiskers at the top and bottom of the boxes indicate the 10th and 90th percentile. The 5th and 95th percentile is indicated by open circles. The means (± SD) of the two parental lines B73 and Mo17 are indicated by closed circles and triangles, respectively.
Effect of temperature at night

Table 2: Main characteristics of QTLs for PSII operating efficiency \( (F_a/F_m') \), maximum quantum efficiency of PSII photochemistry \( (F_v/F_m) \), minimal fluorescence \( (F_o) \), maximal fluorescence \( (F_m) \) and leaf greenness (SPAD) with an LOD score above a threshold of \( \alpha < 0.05 \) identified in the IBM302 population grown under different temperature regimes.

<table>
<thead>
<tr>
<th>Trait</th>
<th>°T</th>
<th>Chr.</th>
<th>cM</th>
<th>Range</th>
<th>Nearest marker</th>
<th>LOD</th>
<th>R²</th>
<th>Add.</th>
<th>Bootstrap LOD (cM)</th>
<th>Joint LOD (cM)</th>
</tr>
</thead>
<tbody>
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<td>( F_a/F_m' )</td>
<td>24/22 °C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17/13 °C</td>
<td>5</td>
<td>254</td>
<td>240-265</td>
<td>php15024</td>
<td>25.86</td>
<td>23.0</td>
<td>-0.051</td>
<td>21.3 (255)</td>
<td>19.2 (255)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>161</td>
<td>147-193</td>
<td>uaz187</td>
<td>8.75</td>
<td>6.3</td>
<td>0.028</td>
<td>7.2 (168)</td>
<td>4.8 (161)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17/6 °C</td>
<td>5</td>
<td>257</td>
<td>249-265</td>
<td>ufg60</td>
<td>8.90</td>
<td>9.2</td>
<td>-0.021</td>
<td>8.9 (255)</td>
<td>11.9 (257)</td>
<td></td>
</tr>
<tr>
<td>( F_v/F_m )</td>
<td>24/22 °C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17/13 °C</td>
<td>5</td>
<td>255</td>
<td>246-271</td>
<td>php15024</td>
<td>18.87</td>
<td>18.3</td>
<td>-0.025</td>
<td>20.0 (257)</td>
<td>15.9 (257)</td>
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<td>9</td>
<td>411</td>
<td>399-432</td>
<td>mmpl132</td>
<td>6.19</td>
<td>6.4</td>
<td>-0.014</td>
<td>6.3 (409)</td>
<td>3.2 (420)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17/6 °C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( F_o )</td>
<td>24/22 °C</td>
<td>6</td>
<td>395</td>
<td>393-408</td>
<td>umc2170</td>
<td>9.35</td>
<td>9.7</td>
<td>0.005</td>
<td>5.3 (393)</td>
<td>7.3 (397)</td>
</tr>
<tr>
<td>7</td>
<td>424</td>
<td>416-430</td>
<td>umc1490</td>
<td>11.97</td>
<td>9.3</td>
<td>0.006</td>
<td>7.5 (422)</td>
<td>15.0 (424)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>250</td>
<td>242-259</td>
<td>umc1457</td>
<td>23.55</td>
<td>19.6</td>
<td>-0.008</td>
<td>13.3 (250)</td>
<td>18.0 (249)</td>
<td></td>
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<tr>
<td>17/13 °C</td>
<td>5</td>
<td>255</td>
<td>249-265</td>
<td>php15024</td>
<td>20.80</td>
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<td>0.039</td>
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<td>umc2026</td>
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<td>6.3</td>
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<td>0.2 (351)</td>
<td></td>
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<tr>
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<td>483</td>
<td>472-487</td>
<td>apg2</td>
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<td>159</td>
<td>144-171</td>
<td>bnl1094</td>
<td>9.08</td>
<td>7.2</td>
<td>-0.024</td>
<td>5.4 (165)</td>
<td>2.9 (132)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17/6 °C</td>
<td>5</td>
<td>254</td>
<td>246-261</td>
<td>php15024</td>
<td>12.47</td>
<td>12.5</td>
<td>0.028</td>
<td>8.1 (255)</td>
<td>1.9 (255)</td>
<td></td>
</tr>
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<td>6</td>
<td>487</td>
<td>483-492</td>
<td>umc2059</td>
<td>9.08</td>
<td>7.9</td>
<td>0.022</td>
<td>5.7 (486)</td>
<td>1.4 (487)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( F_m )</td>
<td>24/22 °C</td>
<td>6</td>
<td>422</td>
<td>414-436</td>
<td>umc1490</td>
<td>10.19</td>
<td>13.7</td>
<td>0.033</td>
<td>4.2 (422)</td>
<td>9.4 (426)</td>
</tr>
<tr>
<td>7</td>
<td>250</td>
<td>242-259</td>
<td>umc1457</td>
<td>18.63</td>
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<td>18.7 (250)</td>
<td></td>
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<tr>
<td>17/13 °C</td>
<td>6</td>
<td>424</td>
<td>418-440</td>
<td>umc1490</td>
<td>9.15</td>
<td>8.5</td>
<td>0.048</td>
<td>3.2 (426)</td>
<td>6.6 (424)</td>
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<td>453</td>
<td>445-455</td>
<td>umc1350</td>
<td>6.83</td>
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<td>165-188</td>
<td>mmpl111</td>
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<td>2.1 (168)</td>
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<td>7</td>
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<td>umc116a</td>
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<td>7.1 (180)</td>
<td>3.1 (209)</td>
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<tr>
<td>SPAD</td>
<td>24/22 °C</td>
<td>2</td>
<td>306</td>
<td>286-323</td>
<td>mmpl119</td>
<td>6.37</td>
<td>6.6</td>
<td>-0.9</td>
<td>8.2 (308)</td>
<td>0.8 (305)</td>
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<tr>
<td>17/13 °C</td>
<td>5</td>
<td>263</td>
<td>228-267</td>
<td>bnl1902</td>
<td>15.80</td>
<td>16.1</td>
<td>-1.2</td>
<td>14.2 (263)</td>
<td>9.5 (263)</td>
<td></td>
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<tr>
<td>5</td>
<td>289</td>
<td>276-315</td>
<td>bnl1208</td>
<td>6.56</td>
<td>5.1</td>
<td>-0.8</td>
<td>5.0 (287)</td>
<td>10.3 (306)</td>
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<td></td>
</tr>
<tr>
<td>17/6 °C</td>
<td>4</td>
<td>373</td>
<td>357-389</td>
<td>umc66</td>
<td>9.38</td>
<td>10.7</td>
<td>1.1</td>
<td>7.2 (368)</td>
<td>5.4 (368)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>218</td>
<td>188-233</td>
<td>umc1447</td>
<td>6.26</td>
<td>6.4</td>
<td>-0.8</td>
<td>8.4 (220)</td>
<td>2.5 (230)</td>
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<td></td>
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<tr>
<td>7</td>
<td>139</td>
<td>128-169</td>
<td>gta101a</td>
<td>6.23</td>
<td>5.6</td>
<td>0.8</td>
<td>7.4 (139)</td>
<td>1.1 (161)</td>
<td></td>
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</tbody>
</table>

*°T = Growth regime, Chr. = Chromosome number, cM = Position of the peak of the QTL in centimorgan, Range = Range was defined by the positions on the chromosome where the LOD score at the QTLs peaks decreased by half, \( R^2 \) = Percentage of the phenotypic variance explained by genotype class at LOD peak, Add. = Additivity (positive additivity = high values of the trait were inherited from Mo17; negative additivity = high values of the trait were inherited from B73); Bootstrap (cM) = LOD score in the bootstrap analysis including the position of the peak in centimorgan, Joint (cM) = LOD score in the joint analysis of both replications including the position of the peak in centimorgan.

of the IBM302 RI population was carried out (Table 2). Figure 2 presents a map of the LOD scores. In total, 28 QTLs were detected above the LOD threshold of \( \alpha < 0.05 \), which was empirically determined by permutation test for eachtrait at each environment and corresponded to an LOD score of around 6. Most of these QTLs could be confirmed by bootstrap analysis and joint analysis of both repetitions (Table 2). The amount of QTLs was higher in seedlings grown at suboptimal temperature, especially when they developed at 17/13 °C, than in plant grown at 24/22 °C. Whilst QTLs were found for all photosynthesis-related traits, the analysis did not reveal any significant QTL for the rate of shoot growth in the three temperature regimes. However, some peaks with an LOD around 4.0 to 4.5 were detected (Figure 2).

In plants grown at 17/13 °C, the PSII efficiencies \( F_a/F_m' \) and \( F_v/F_m \) as well as the minimal fluorescence \( (F_o) \) were mainly controlled by a QTL, which was detected on chromosome 5 at ~255 cM. It explained about 20% of the phenotypic variance for these traits. The indication of additivity showed that Mo17 carried the favorable allele at this locus. This QTL was also present in seedlings that were raised at 17/6 °C, but additive effects and phenotypic variance were considerably smaller compared to the situation in seedlings grown at 17/13 °C. In plants grown at 24/22 °C, this QTL was not present.

On chromosome 6 between 420 and 490 cM, probably two independent QTLs were present, which were responsible for the genotypic variations of the investigated chlorophyll fluorescence parameters. For both QTLs, the favorable allele was inherited by Mo17.
The QTL at 424 cM was found in seedlings grown at optimal and at suboptimal temperature. In contrast, the QTL at about 480 cM was present only in plants that developed at suboptimal temperature. On chromosome 8 at 250 cM, a strong QTL was detected for the fluorescence levels $F_m$ and $F_o$ in plants grown at 24/22 °C, but not in plants grown at suboptimal temperature. This QTL explained about 20% of the phenotypic variance. High trait values were inherited from Mo17.

Identification and testing of candidate genes
Potential candidate genes were searched for the major QTL on chromosome 5. The positions of genes that are located in the QTL regions were obtained from the Maize Genetics and Genomics Database (http://www.maizegdb.org). Based on their position and their presumable function in the response to chilling stress, the invertase gene ivr2 and the locus anthocyaninless2 (a2), coding for an anthocyanidin synthase, were identified as potential candidate genes. In order to examine whether the gene of one of these enzymes is responsible for the expression of the QTL on chromosome 5, the activity of soluble acid invertase and the amount of anthocyanin was analyzed in the RI lines of the IBM302 population grown at suboptimal (17/13 °C) and optimal temperature (24/22 °C) and QTL analyses were conducted using an LOD threshold of $\alpha = 0.1$ determined by permutation test.

The activity of soluble acid invertase was lower in seedlings grown at 17/13 °C than in plants that developed at 24/22 °C, in average 0.47 versus 0.58 µmol m$^{-2}$ s$^{-1}$ in the RI lines. The QTL analysis revealed that the QTL region on chromosome 5 was not involved in genotypic differences of invertase activity, although the QTL for PSII efficiency was verified in this experiment using different equipment for chlorophyll fluorescence analysis (data not shown). However, two major QTLs for soluble acid invertase activity were detected on chromosome 2 and 3 in seedlings grown at suboptimal temperature.

Table 3: Main characteristics of QTLs for soluble acid invertase activity (µmol m$^{-2}$ s$^{-1}$) and anthocyanin content (µmol m$^{-2}$) with an LOD score above a threshold of $\alpha < 0.1$ identified in the IBM302 population grown under different temperature regimes.

<table>
<thead>
<tr>
<th>Trait</th>
<th>°T</th>
<th>Chr.</th>
<th>cM</th>
<th>Range</th>
<th>Nearest marker</th>
<th>LOD</th>
<th>$R^2$</th>
<th>Add.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invertase</td>
<td>24/22 °C</td>
<td>2</td>
<td>205</td>
<td>191-209</td>
<td>mmp42</td>
<td>5.96</td>
<td>8.6</td>
<td>-0.079</td>
</tr>
<tr>
<td></td>
<td>17/13 °C</td>
<td>2</td>
<td>205</td>
<td>198-211</td>
<td>mmp42</td>
<td>15.18</td>
<td>14.2</td>
<td>-0.091</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>553</td>
<td>539-563</td>
<td>umc1273</td>
<td></td>
<td>10.90</td>
<td>12.3</td>
<td>-0.082</td>
</tr>
<tr>
<td>Anthocyanin</td>
<td>24/22 °C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>17/13 °C</td>
<td>10</td>
<td>317</td>
<td>305-330</td>
<td>bnl10.13a</td>
<td>11.07</td>
<td>18.0</td>
<td>-1.36</td>
</tr>
</tbody>
</table>

°T = Growth regime, Chr. = Chromosome number, cM = Position of the peak of the QTL in centimorgan, Range = Range was defined by the positions on the chromosome where the LOD score at the QTLs peaks decreased by half, $R^2$ = Percentage of the phenotypic variance explained by genotype class at LOD peak, Add. = Additivity (positive additivity = high values of the trait were inherited from B73; negative additivity = high values of the trait were inherited from Mo17)
(Table 3). Although the QTL for invertase activity at chromosome 2, which was detected also at optimal temperature, was more significant at 17/13 °C than at 24/22 °C, it did not co-localize with QTLs for chilling-tolerance of photosynthesis. This rules out that the ivr2 gene is involved in protection from chilling stress under the studied conditions.

The analysis of the anthocyanin content in the IBM302 population showed that suboptimal temperature induced an accumulation of anthocyanin. In the RI lines, the anthocyanin content was in average 3.0 µmol m⁻² at 24/22 °C and 8.0 µmol m⁻² at 17/13 °C. The QTL analysis revealed a major QTL for the content of anthocyanin in seedlings grown at suboptimal temperature, which, however, was not localized on chromosome 5, but on chromosome 10 near marker bnl.10.13a (Table 3). No significant QTL for the content of anthocyanin was detected in plants that developed at optimal temperature.

**Discussion**

Our study aimed to elucidate the effect of temperature at night on the chilling acclimation of maize seedlings and to determine its genetic basis by QTL analyses. The decrease of the temperature, from 24/22 °C to 17/13 °C, caused a reduction of the photosynthetic performance, especially to a low PSII operating efficiency ($F_{q'/q}$) in genotype B73. The decrease of $F_{q'/q}$ in genotype B73 was less pronounced when the temperature at night was 6 °C. This genotype dependent effect of the temperature at night on the photosynthetic performance was also disclosed by the comparison of QTLs from both low temperature stress conditions (17/13 °C and 17/6 °C). Even though there were no specific QTLs for $F_{q'/q}$ and $F_{m'/m}$ at 17/6 °C, the additivity of the main QTL at ~255 cM on chromosome 5 of plants grown at 17/13 °C was reduced by half at 17/6 °C. Due to the lack of QTLs expressed solely at low temperature at night (17/6 °C), but not at 17/13 °C, the QTL on chromosome 5 seems to be the major factor explaining the lower PSII efficiency of B73 than Mo17 at 17/13 °C whilst both genotypes exhibited a similar $F_{q'/q}$ at 17/6 °C. However, this does not explain why $F_{q'/q}$ in B73 was lower at 17/13 °C than at 17/6 °C. Similar to the response of B73, a lower temperature at night had a positive effect on photosynthesis in the C₄ grass Muhlenbergia montana (Pittermann and Sage, 2001); a lower CO₂ assimilation rate was found in plants grown at 24/16 °C than at 24/4 °C, in particular when photosynthesis was measured at high temperature. The physiological cause of this behavior still remains to be elucidated. Concerning the studied genotypes, it appears that an unknown factor reduces the PSII operating efficiency in both genotypes at 17/13 °C compared to 17/6 °C, however, the Mo17 allele at the QTL on chromosome 5 seems to allow this genotype to better compensate for the loss of photosynthetic efficiency. The major QTL on the short arm of chromosome 5 at about 255 cM was detected for most of the studied traits ($F_{q'/q}$, $F_{m'/m}$, $F_{a'b}$ and SPAD) in plants grown at 17/13 °C and showed a considerable phenotypic variance with regard to the chlorophyll fluorescence parameters. Therefore, it is considered to be a major locus for chilling tolerance of photosynthesis in this population. Based on their position and their potential involvement in chilling acclimation, the invertase gene $ivr2$ and the locus anthocyaninless2 (a2), coding for an anthocyanidin synthase, were identified as candidate genes for this QTL. Furthermore, the EST $p$-csu774, which shows high similarity with the maize chlorophyll a/b binding protein 6A mRNA, is present in this region. However, this EST is located more proximal from the QTL position for $F_{q'/q}$ and $F_{m'/m}$; it lays closer to the peak of the QTL for leaf greenness (SPAD) at 263 cM, which was identified in seedlings grown at 17/13 °C. The rational for choosing the $ivr2$ gene, which codes for the vacuolar acid invertase 2, as potential candidate gene was that the vacuolar acid invertase is involved in the soluble sugar metabolism and has been found to be induced in maize under drought stress conditions (Pellessi et al., 1999). Furthermore, a cold-induced increase of the activity of acid invertase has been observed in wheat leaves (Vargas et al., 2007). On one side, the stress-responsiveness of invertase seems to be based on the fact that glucose, which is the product of the sucrose cleavage by invertase, acts as signaling molecule capable to induce stress-responsive genes; on the other side, invertase plays a central role in the overall adjustment of carbohydrate metabolisms, which is usually strongly affected when plants are exposed to stress conditions (Roitsch and González, 2004). However, analysis of soluble acid invertase in the IBM302 population showed that its activity is lower in seedlings grown at suboptimal than in plants that developed at optimal temperature, indicating a minor role of soluble acid invertase in the chilling-response of maize. Moreover, the QTL analysis of the IBM302 population has shown that there is no QTL for soluble acid invertase activity on chromosome 5 and no co-localization of QTLs for chilling-tolerance of photosynthesis and activity of invertase. Instead, a major QTL for soluble acid invertase activity was found on chromosome 2 at marker $mpp42$. Aligning the genetic map of the IBM302 population with the IBM2 2008 Neighbors map showed that this marker is only 11 cM away from the $ivr1$ gene. This makes it very likely that $ivr1$ underlies the QTL for invertase at chromosome 2. Consequently, the IBM302 mapping population seems to be of high value for further analysis of the genetic control of the $ivr1$ locus and...
the function of its gene product. This is underlined by the fact that this genomic region harbors QTLs affecting amylopectin and starch content in another maize mapping population (Séne et al., 2000). Equal to ivr2, the locus anthocyaninless2 (a2) is directly located at the position of the major QTL on chromosome 5. The a2 gene, which codes for anthocyanidin synthase, a key enzyme of the anthocyanin synthesis, was found to be strongly induced under cold (Christie et al., 1994). However, the QTL analysis revealed no significant QTL for anthocyanin content in the region of the major QTL for chilling tolerance of photosynthesis, ruling out that a2 is the underlying gene of the QTL for photosynthesis-related traits on chromosome 5. As for the locus a2, the positions of several further genes involved in the accumulation of anthocyanin are known. Among these genes the locus r1, which shares sequence similarity with the MYC class of DNA binding proteins (Chandler et al., 1989) and which is known to be induced by cold (Christie et al., 1994), is closely located to the QTL found for the content of anthocyanin on chromosome 10 and might be, therefore, the underlying gene. Since this QTL for the content of anthocyanin was not associated with a QTL for chilling tolerance of photosynthesis, the role of anthocyanin in the acclimation to suboptimal temperature seems to be insignificant, at least in the investigated maize material and under the studied conditions.

The conservation of QTLs in different mapping populations exposed to similar stress conditions indicates the importance of specific genomic regions in the plant’s response to stress. The QTLs found in IBM302 were compared with QTLs for chilling tolerance described in other studies and mostly in other mapping populations (Fracheboud et al., 2002; Fracheboud et al., 2004; Jompuk et al., 2005; Presterl et al., 2007; Rodríguez et al., 2008). The analysis revealed some co-localizations of QTLs between these populations. A QTL for leaf chlorosis and frost damage, which was identified in the SL × TH dent mapping population grown under field conditions in Germany and France (Presterl et al., 2007), overlapped with the major QTL on chromosome 5 found in the IBM302 population. No co-localization were found between the QTLs detected in the present study and QTLs found under similar growth conditions in the Ac7643 × Ac7729 population (Fracheboud et al., 2002) and in the IBM302 population, which was, however, examined at an earlier growth stage (Rodríguez et al., 2008). By comparing the present results with the QTLs detected in the ETH-DL3 × ETH-DH7 mapping population (Fracheboud et al., 2004; Jompuk et al., 2005), two chromosomal regions were found, which showed an overlap of QTLs for similar traits. One of these chromosomal regions is located on chromosome 8 and was found to be significantly involved in the level of fluorescence (F₀ and Fₙ) at optimal temperature in the IBM302 population and, in the ETH-DL3 × ETH-DH7 population, in F₀ at 15 °C (Fracheboud et al., 2004) and in Fₙ at cool field conditions (Jompuk et al., 2005). The major QTL for chilling tolerance of photosynthesis in the ETH-DL3 × ETH-DH7 population, which was detected in the telomeric region of the long arm of chromosome 6, seems to be located in the same region as the QTLs for F₀ and Fₙ found in the IBM302 population. Re-analysis of the data from Fracheboud et al. (2004) with an improved genetic map and alignment of the genetic maps of chromosome 6 from the ETH-DL3 × ETH-DH7 and the IBM302 population showed that the QTL for F₀, which was found in the IBM302 population grown at optimal temperature, corresponded to the QTL for F₀ (and other photosynthesis-related traits) found in the ETH-DL3 × ETH-DH7 population grown at suboptimal temperature (Figure 3). The re-analysis of the ETH-DL3 × ETH-DH7 data revealed a second QTL close to the telomere that was in the same chromosomal region as the QTL for F₀ found in the IBM302 population grown at suboptimal temperature (17/13 °C and 17/6 °C). The parameter F₀ is of great interest, since it is taken as a good indicator of the integrity of the

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**Figure 3:** Comparison of conservation of QTLs for the minimal fluorescence (F₀) on the telomeric region of the long arm of chromosome 6 in the IBM302 and ETH-DL3 × ETH-DH7 populations. The position (in cM) and the markers are given in the maps. Matching markers between populations are connected by lines. The gray scale bar show the range LOD values from ≤ 0 (white) to ≥ 15 (black).
PSII reaction center (Baker, 2008). Consequently, it demonstrates that this QTL region seems to be involved in the development of a functional photosynthetic machinery under low temperature.

A potential candidate gene for this QTL region was discussed in Jompuk et al. (2005), which were agp2 coding for the small subunit of leaf ADP glucose pyrophosphorylase. However, a re-analysis of the genetic localization of this gene has revealed that it is positioned on chromosome 1 (data not shown). Furthermore, due to the strong expression of this QTL for traits reflecting the integrity of the photosynthetic apparatus other genes, which seem to be directly involved in the photosynthetic light reaction, are more likely to reflect the molecular cause of this QTL. Based on the previous and the present results, the search for candidate genes in the vicinity of bnlg1740, which could explain the observed phenotype, revealed cab-m7 as a potential candidate gene. Cab-m7 codes for the LHCII protein Lhcbm7 and is preferentially expressed in maize mesophyll cells and is strongly induced upon illumination (Becker et al., 1992). Co-localization of Lhcb genes with QTLs for cold-induced photoinhibition was also reported in another study of maize QTLs (Pimentel et al., 2005).

It is noteworthy that, although both B73 and Mo17 are not well adapted to chilling conditions, it was possible to find common QTLs with other mapping populations but at a much higher resolution, in contrast to the Swiss dent population (ETH-DL3 × ETH-DH7), in which one of the parents are characterized by a high chilling tolerance. The comparison between cold-adapted and non-adapted genotypes may assist us in detecting genomic regions that have not yet been improved in the maize plant for adaptation to chilling stress. These regions could then be considered for a more exhaustive screening of different maize genotypes to obtain the best alleles in maize. Moreover, comparative mapping with more cold tolerant plant species, such as barley or wheat, would enable the identification of possible candidate genes for gene transfer to elite maize genotypes.

This study brings new insights into the complexity of studying the tolerance of maize to chilling stress. This is emphasized by the different genetic responses of photosynthetic traits when plants are exposed to different temperatures at night. Therefore, low temperature at night must be considered when studying the acclimation of the photosynthetic apparatus of maize to chilling stress. The comparative QTL analysis of different mapping populations revealed chromosomal regions, which seems to be important for future research. Determining the gene(s) that explain the QTLs is relevant for a better understanding of adaptation of maize to chilling stress and to the production of new genotypes with improved tolerance to chilling stress.
Chapter 9

Genetic analysis of antioxidants and C₄ cycle enzymes in maize seedlings grown at suboptimal temperature*

The primary cause of genotypic differences in chilling sensitivity of maize seedlings is under dispute. The involvement of C₄ cycle enzymes and antioxidants in the chilling tolerance of maize photosynthesis has been discussed for many years. To unravel the importance of C₄ cycle enzymes and antioxidants for the chilling tolerance of photosynthesis, quantitative trait loci (QTLs) for the amounts of antioxidants and the activities of the C₄ cycle enzymes were mapped in maize seedlings of the ETH-DL3 × ETH-DH7 population grown at suboptimal temperature; the identified QTLs were then compared with QTLs for chlorophyll content and for photosynthesis-related traits from an earlier study. Significant QTLs were detected for the extractable activity of phosphoenolpyruvate carboxylase (EC 4.1.1.31), malate dehydrogenase (EC 1.1.1.37) and malic enzyme (EC 1.1.1.38) as well as for the amounts of ascorbate and α-tocopherol. These QTLs, however, were not co-localized with the major QTLs for photosynthesis-related traits, indicating that the C₄ cycle enzymes and the studied antioxidants play a minor role in the genetic difference in chilling tolerance of photosynthesis. Based on the QTL analysis, a strong relationship was found between the chlorophyll a/b ratio and the chilling tolerance of photosynthesis. The underlying mechanisms of this pleiotropic effect as well as potential candidate genes are discussed.

Introduction

Maize (Zea mays L.) is a crop of tropical/subtropical origin and is characterized by a low chilling tolerance. In temperate regions, the high temperature requirement of maize is often unfulfilled during the transition from heterotrophic to autotrophic growth when maize is most sensitive to chilling (Stamp, 1984). A primary cause for this is not yet known, probably due to the fact that low temperature affects a wide range of processes in the maize seedling (for reviews see Leipner and Stamp, 2009; Marocco et al., 2005). Among the various effects of low temperature on the physiology of maize, high susceptibility of the photosynthetic apparatus seems to be of particular importance (Baker et al., 1994). However, the mechanism responsible for the reduction of leaf photosynthesis at low temperature is under discussion; reduced activity of photosynthetic enzymes (Kingston-Smith et al., 1997), reduced photosynthetic efficiency and capacity (Haldimann et al., 1996), disturbance of chloroplast development (Nie and Baker, 1991) and alterations in the phloem loading (Sowinski et al., 2001) have all been proposed. Special attention has been paid to the enzymes of the C₄ cycle due to the high susceptibility of C₄ plants to chilling (Long, 1983). In particular, re-synthesis of phosphoenolpyruvate has been found to be the limiting factor of C₄ photosynthesis, especially at low temperature (Laisk and Edwards, 1997). Besides the processes that are directly involved in photosynthesis, the antioxidative systems were studied extensively based on the justified assumption that greater amounts of reactive oxygen species are generated during low temperature stress (for a review see Foyer et al., 2002).

There have been many studies in which genotypes of contrasting chilling tolerance were characterized. It is accepted that chilling-sensitive genotypes have high amounts of zeaxanthin, probably due to a greater requirement for dissipative mechanisms caused by their low photosynthetic performance at suboptimal temperature (Haldimann, 1998). The picture is, however, less clear with regard to other processes, which may be involved in the response of maize seedlings to chilling stress. Enzymes of the C₄ cycle were studied by comparing C₄ species with different degrees of tolerance to chilling; it was found that there was an association of the pyruvate orthophosphate dikinase (PPDK) with the chilling tolerance of photosynthesis (Usuda et al., 1984a). In chilling-stressed maize, a higher activity of malate dehydrogenase (MDH) was found in cold-adapted material compared to a genotype from tropical lowlands (Stamp, 1987a). Genotypic differences in phosphoenolpyruvate carboxylase (PEPC) activity seem to be small (Pietrini et al., 1999; Stamp, 1987a). There does not seem to be a clear correlation between the level of antioxidants in plants grown at optimal temperature or after a short-term chilling stress with the chilling tolerance of the genotype (Hodges et al., 1996). However, the con-

* based on the following publication:
tent of glutathione in seedlings grown under chilling stress seems to be related to the chilling tolerance of the genotype, as was found under controlled conditions (Kocsy et al., 1996) and in the field (Leipner et al., 1999a).

In studies of interactions among different traits, a comparison of a few genotypes holds the risk that coincidental associations may be detected, which do not have a common genetic basis. The quantitative trait locus (QTL) analysis is a powerful tool for studying the interaction between complex traits and for assigning such interactions to a particular genomic region (Vreugdenhil et al., 2007). There have been several QTL studies on the chilling tolerance of photosynthesis of maize (Fracheboud et al., 2002; Fracheboud et al., 2004; Jompuk et al., 2005; Pimentel et al., 2005). In the ETH-DL3 × ETH-DH7 mapping population, which was developed from a chilling-tolerant and a chilling-sensitive dent maize line, major QTLs involved in the chilling tolerance of seedlings were stable in cold environments (Jompuk et al., 2005). Consequently, this mapping population is very useful for unraveling the interaction among chilling tolerance of photosynthesis, activity of C₄ cycle enzymes and level of antioxidants.

If antioxidants or C₄ cycle enzymes are directly involved in the chilling tolerance of photosynthesis in maize seedlings, then a co-localization for QTLs of these traits and for QTLs for photosynthesis-related traits must be found. To prove this hypothesis, a QTL analysis was conducted for the amounts of major traits and for QTLs for photosynthesis-related traits in maize seedlings, then a co-localization for QTLs of chilling tolerance of photosynthesis, activity of C₄ cycle enzymes and level of antioxidants.

Materials and methods

Plant material and growth conditions

Maize (Zea mays L.) lines with contrasting chilling-tolerance of photosynthesis were obtained by divergent selection from a Swiss dent maize breeding population based chlorophyll fluorescence analysis (Fracheboud et al., 1999). Two lines in the S₅ generation, namely ETH-DH7 (chilling-tolerant) and ETH-DL3 (chilling-sensitive), were used as parents to produce a segregating F₂ population. The genetic linkage map was constructed from 254 F₂ plants by means of simple sequence repeat markers (Fracheboud et al., 2004). The resulting F₂:₃ population with 226 families was used in the QTL experiments described here.

The plants were grown in a growth chamber (PGW36, Conviron, Winnipeg, Canada) in 0.75 l pots containing a mixture of soil, peat and compost (Topf und Pikiererde 140, Ricoter, Aarberg, Switzerland). Plants were first grown for six days at 25/22 °C (day/night), a photoperiod of 12 h at 400 µmol m⁻² s⁻¹ (Sylvania CW/VHO, Osram Sylvania, Mississauga, Canada) and a relative humidity of 60/70 % (day/night) followed by 14 days at 15/13 °C (day/night); the other conditions were not changed. The 226 families were grown at intervals in blocks of six plants of 11 families. Leaf discs, 0.48 cm², were taken from the middle part of the third fully developed leaf of each plant, pooled for each family and stored at -80 °C until analysis.

Assay of α-tocopherol and chlorophyll

Six frozen leaf discs were homogenized (Retsch Schwingmühle MM2, Haan, Germany) for 1 minute and extracted in 1 ml ice-cold 80 % (v/v) acetone. The sample was centrifuged (10 minutes, 10000 g, 4 °C). An aliquot of 200 µl was used to determine the chlorophyll concentration according to Arnon (1949). To assay α-tocopherol, 500 µl of the extract was mixed with 500 µl hexane and 2500 µl H₂O. After centrifugation (3 minutes, 150 g, 4 °C) the hexane layer was collected and the water phase re-extracted with 500 µl hexane. The pooled hexane fraction was evaporated under vacuum, re-suspended in 1000 µl methanol and separated by HPLC (Jasco, Tokyo, Japan) according to Leipner et al. (2000b). Isocratic separation was done on a Supelcosil LC-18 column (4.6 × 150 mm, 3 µm particle size) with 50:40:1 of methanol:acetoni-trile:N-acetate-buffer (0.5 M, pH 4.9) from 0 to 12 minutes and ethylacetate from 12 to 13 minutes. The column was re-equilibrated for 5 minutes. The injection volume was 20 µl and the flow rate 1.2 ml min⁻¹. Detection of α-tocopherol was made at +0.5 V with an amperometric detector cell with a glassy carbon electrode and an Ag/AgCl reference electrode (LC-44, BAS Technicol, Congleton, U.K.). Quantification was done by peak area integration using data acquisition software (JCL6000, Jones Chromatography, Hen-goed, U.K.). The α-tocopherol peak was identified according to its retention time compared to the standard (Sigma).

Assay of ascorbate and glutathione

Six frozen leaf discs were homogenized (Retsch Schwingmühle MM2, Haan, Germany) for 1 minute and extracted in 1.5 ml ice-cold extraction buffer (40 % [v/v] methanol; 0.75 % [w/v] meta phosphoric acid; 16.7 mM oxalic acid; 0.127 mM DTPA). The extract was centrifuged for 5 minutes at 10000 g (4 °C), and 100 µl of the supernatant were transferred to 900 µl of the mobile phase (20.5 mM citric acid; 3.1 mM Na₂HPO₄; 40.7 µM DTPA; 60 % [v/v] methanol; 0.03 % [v/v] octyl amine). Isocratic separation was made on a Supelcosil LC-NH₂ column (4.6 × 250 mm, 5 µm particle size). The injection volume was 20 µl and the flow rate 0.8 ml min⁻¹. Detection
was made at +0.8 V using an amperometric detector cell with a gold electrode and an Ag/AgCl reference electrode (LC-44, BAS Technicoll, Congleton, U.K.). Peaks were quantified by peak area integration with data acquisition software (JCL6000, Jones Chromatography, Hengoed, U.K.). Ascorbate and glutathione were identified according to their retention time compared to the standard (Sigma).

Enzyme assay
Six frozen leaf discs were homogenized (Retsch Schwingmühle MM2, Haan, Germany) for 1 minute and extracted in 2 ml ice-cold extraction buffer (50 mM K phosphate buffer, pH 8.0; 10 mM MgCl2; 5 mM NaHCO3; 2.5 mM phosphoenolpyruvate, 5 units malate dehydratase and 0.2 mM NADH.

Malate dehydrogenase (MDH, EC 1.1.1.37) was determined by monitoring the decrease in NADH concentration at 340 nm (Usuda et al., 1984b). The reaction mixture contained 50 mM Hepes-KOH (pH 8.0), 5 mM pyruvate, 2.5 mM KH2PO4, 10 mM MgCl2, 5 mM dithiothreitol (DTT), 10 mM malic acid, 56.25 mM MgCl2 and 0.5 mM NADPH. Malic enzyme (ME, EC 1.1.1.38) was assayed by following the decrease in the absorbance of NADPH at 340 nm, the samples were incubated in the absence of pyruvate orthophosphate dikinase (PPDK). The concentrations that of malate dehydrogenase (MDH) and pyruvate orthophosphate dikinase (PPDK). The concentrations decreased to 0.275 units ml-1 PEPC and 1.25 mM ATP. Prior to determination of pyruvate orthophosphate dikinase (PPDK), the activity of phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), malic enzyme (ME) was higher than that of malate dehydrogenase (MDH) and a lower content of glutathione compared to the chilling-sensitive genotype ETH-DL3. On average, the F2:3 population had an activity of the C4 cycle enzymes and amounts of antioxidants that were at the level of the parental means or at the level of the best parent. Of the C4 cycle enzymes, the activity of phosphoenolpyruvate carboxylase (PEPC) and, especially, malic enzyme (ME) was higher than that of malate dehydrogenase (MDH) and pyruvate orthophosphate dikinase (PPDK). The concentrations.

Quantitative trait loci analysis
The QTL analysis was performed with QTL Cartographer v. 1.17b software (Basten et al., 1994). Composite interval mapping with model 6 of the Zmapqtl program module was used to map the QTLs and estimate their effects (Basten et al., 2005). The genome was scanned at 2-cM intervals, and the window size was set at 30 cM. Cofactors were chosen according to the forward-backward method of stepwise regression at p(Fst) = p(Fout) = 0.05. The presence of a QTL was declared significant when the LOD value was higher than 2.5. Additive effects of the detected QTLs were also estimated by the Zmapqtl procedure of QTL Cartographer (Basten et al., 2005); the resulting R2 value (coefficient of determination) indicated the percentage of phenotypic variance explained by marker genotypes at the locus.

Results
With a few, but important exceptions, there were no major differences among the parental lines with respect to the activity of the C4 cycle enzymes or to the content of the antioxidants (Table 1). However, the chilling-tolerant genotype ETH-DH7 was characterized by a remarkably higher activity of malate dehydrogenase (MDH) and a lower content of glutathione compared to the chilling-sensitive genotype ETH-DL3. On average, the F2:3 population had an activity of the C4 cycle enzymes and amounts of antioxidants that were at the level of the parental means or at the level of the best parent. Of the C4 cycle enzymes, the activity of phosphoenolpyruvate carboxylase (PEPC) and, especially, malic enzyme (ME) was higher than that of malate dehydrogenase (MDH) and pyruvate orthophosphate dikinase (PPDK). The concentrations.

Table 1: Extractable activity of phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), malic enzyme (ME) and pyruvate phosphate dikinase (PPDK) and content of antioxidants, glutathione and α-tocopherol of the parental lines and the 226 F2:3 families from their cross grown at suboptimal temperature. Values are means ± SD of 5 (parental lines) and 226 (F2:3 families) replications, respectively. Significant differences between the parental lines are indicated by * = P < 0.05, (•) = P < 0.1 and ns = not significant.

<table>
<thead>
<tr>
<th>Trait</th>
<th>ETH-DH7</th>
<th>ETH-DL3</th>
<th>F2:3</th>
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<tr>
<td>C4 cycle enzymes</td>
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<tr>
<td>PEPC</td>
<td>55.6 ± 5.7</td>
<td>56.0 ± 11.4</td>
<td>NS 66.3 ± 21.7</td>
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<tr>
<td>MDH</td>
<td>50.6 ± 6.1</td>
<td>30.1 ± 8.2</td>
<td>* 48.1 ± 20.7</td>
</tr>
<tr>
<td>ME</td>
<td>81.3 ± 16.3</td>
<td>70.4 ± 21.9</td>
<td>NS 75.2 ± 20.6</td>
</tr>
<tr>
<td>PPDK</td>
<td>40.4 ± 19.2</td>
<td>44.5 ± 14.6</td>
<td>NS 44.6 ± 24.3</td>
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<tr>
<td>Antioxidants</td>
<td></td>
<td></td>
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<tr>
<td>Ascorbate</td>
<td>1166 ± 192</td>
<td>1093 ± 56</td>
<td>NS 1064 ± 206</td>
</tr>
<tr>
<td>Glutathione</td>
<td>108 ± 8</td>
<td>131 ± 18</td>
<td>(x) 136 ± 50</td>
</tr>
<tr>
<td>Tocopherol</td>
<td>13.2 ± 2.0</td>
<td>14.7 ± 0.3</td>
<td>NS 14.2 ± 4.6</td>
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</table>
(per leaf area) of the antioxidants were 100:10:1 for ascorbate, glutathione and α-tocopherol, respectively. The chlorophyll a+b content was not significantly correlated with the amounts of antioxidants (data not shown). A similar situation was found for most enzymes of the C₄ cycle; however, a significant correlation existed between the chlorophyll content and the activity of the malic enzyme (ME) (r² = 0.207). A significant correlation was found also between the C₄ cycle enzymes ME and PEPC (r² = 0.218). All other correlations were insignificant. The QTL analysis revealed the presence of seven significant QTLs for the C₄ cycle enzymes at six loci (Table 2). Figure 1 gives a map of the LOD scores and also shows the position of the QTLs in relation to the QTLs for photosynthesis-related traits from a previous study under the same growth conditions (Fracheboud et al., 2004). The major QTLs were located on chromosome 1 at 68 and 246 cM for ME activity and on chromosome 8 at 101 cM for MDH activity. For the QTLs for ME activity on chromosome 1, high enzyme activity was due to the allele from ETH-DL3 at 68 cM whilst it was due to the ETH-DH7 allele at 246 cM. The sign of the additivity indicates that the allele for high MDH activity on chromosome 8 (101 cM) was inherited from ETH-DL3. Chromosome 8 harbored another QTL for MDH activity which was located at 23 cM. This QTL was in the region of the gene mdh1 according to an alignment of the QTL position with the position of genes for C₄ cycle enzymes on the IBM2 2005 neighbors map and the Pioneer composite 1999 map. The other QTLs were not located in the vicinity of known genes of C₄ cycle enzymes.

For the antioxidants, four QTLs were found for the ascorbate content and two for the α-tocopherol content (Table 2). No QTL was detected for the glutathione content. The major QTL for ascorbate content was located on chromosome 8 (109 cM), in the same region as one of the QTLs for MDH activity, but with a different sign of additivity. At this position, a high content of ascorbate was inherited from ETH-DH7. A major QTL for α-tocopherol content was found on chromosome 9 (87 cM), whose favorable allele was inherited from ETH-DL3.

An analysis of the chlorophyll content in the F₂:3 population revealed two major chromosomal regions to be important for the chlorophyll content: one on chromosome 2 (~110 cM) and on the telomeric region of the long arm of chromosome 3 (Table 2). The latter probably contained two distinct QTLs at 200 and 230 cM, respectively. The favorable alleles for high chlorophyll content were inherited from ETH-DH7 for the QTL on chromosome 2 and from ETH-DL3 for the QTLs on chromosome 3. Chromosome 6 had a strong QTL for the chlorophyll a+b ratio at 217 cM that was not related to chlorophyll content itself. High chlorophyll a/b ratio was inherited from the chilling-tolerant parent, ETH-DH7.

The comparison of QTLs here with previously found ones for chilling tolerance of photosynthesis (Fracheboud et al., 2004) revealed only few QTL co-localizations (Figure 1). The major QTL for chilling tolerance of photosynthesis, which is located on the telomere of the long arm of chromosome 6, co-localized with a weak QTL for the ascorbate content, which was, however, below the threshold. This region also harbors the QTL for the chlorophyll a/b ratio. A second major QTL for chilling tolerance of photosynthesis is located near the centromere of chromosome 2 and also contained a QTL for ascorbate content as well as the QTL for chlorophyll content. With regard to QTLs for the activity of C₄ cycle enzymes and antioxidants in this study, there are two major QTLs on chromosome 3. Chromosome 3 had a strong QTL on chromosome 2 and also contained a QTL for MDH activity.
enzymes, a co-localization was found on the long arm of chromosome 1 for PEPC and ME activity and for leaf greenness, carbon exchange rate, operating quantum efficiency of PSII ($F_{q'}/F_{m'}$) and maximum quantum efficiency of PSII primary photochemistry ($F_v/F_m$).

**Discussion**

With respect to the cold-induced decrease in the activity of the C₄ cycle, much attention has been paid to pyruvate orthophosphate dikinase (PPDK) as the primary cause of the susceptibility of C₄ plants to chilling (Long, 1983). However, it is not yet known whether the different levels of chilling tolerance of maize genotypes is indeed due to differences in the activity of enzymes of the C₄ cycle. The comparison of the chilling-sensitive parental line ETH-DL3 and the chilling-tolerant ETH-DH7 revealed no differences in the extractable activity of the C₄ cycle enzymes, with the exception of the higher activity of malate dehydrogenase (MDH) in the chilling-tolerant line. Similarly, the activity of MDH was higher in cold-adapted European and tropical highland material compared to a genotype from tropical lowlands, especially under cold stress (Stamp, 1987a). The comparison of genotypes in this and in other studies (Pietrini et al., 1999; e.g. Stamp, 1987a) seems to indicate that differences in the activity of C₄ cycle enzymes frequently exist between genotypes, but that these differences do not necessarily explain the general chilling tolerance of the studied genotype. To unravel the impact of genotypic differences in the activity of C₄ cycle enzymes on the chilling tolerance of photosynthesis, the results of the QTL analysis of the extractable activity of C₄ cycle enzymes were compared with the results of a previous QTL analysis of photosynthesis-related traits under the same growth conditions (Fracheboud et al., 2004). Although the differences in the activity of the C₄ cycle enzymes of the parental lines were slight, several QTLs were detected for enzyme activity, indicating transgressive segregation. However, there were no clear pleiotropic effects between chilling tolerance of photosynthesis, measured by chlorophyll fluorescence technique, and extractable activity of C₄ cycle enzymes. This indicates that the genotypic difference in the chilling tolerance of photosynthesis between ETH-DH7 and ETH-DL3, which is due mainly to allelic variation on chromosomes 2 and 6 (Fracheboud et al., 2004; Jompuk et al., 2005), cannot be explained by differences in the extractable activity of the C₄ cycle enzymes for leaves developed at moderate chilling stress. It seems that the genomic region of these two major QTLs for chilling tolerance of photosynthesis do not harbor genes of the C₄ cycle enzymes. The region of the QTL for photosynthesis-
related traits on chromosome 1, which was found only in plants grown at suboptimal temperature (Fracheboud et al., 2004), contains the gene mdh4 coding for a cytosolic MDH as well as QTLs for ME and PEPC activity. However, it remains to be determined whether this reflects an actual pleiotropic effect or random co-localizations. It is noticeable that there were only few co-localizations of QTLs of the activity of C4 cycle enzymes with the position of the known genes of the C4 cycle enzymes. Other factors may regulate the activity of the enzyme, such as transcription factors or factors involved in the post-transcriptional regulation of the C4 cycle enzymes. Co-localizations between QTLs for photosynthesis-related traits and QTLs for antioxidants were found only for the content of ascorbate, not for glutathione and α-tocopherol. The QTL for the ascorbate content on chromosome 2 had the same map position as one of the major QTLs for photosynthesis in plants grown under the same conditions as in this study (Fracheboud et al., 2004). The gene dar1, which codes a dehydroascorbate reductase, is located in close proximity to this QTL. The direction of additivity of this QTL, however, revealed that F2:3 plants, carrying the allele for higher chilling tolerance of photosynthesis at this position, were characterized by lower ascorbate content. The reason for this pleiotropic effect may be that chilling-sensitive genotypes have a higher requirement for ascorbate and, consequently, it is unlikely that dar1 is the underlying gene of this QTL. The observation that the weak QTLs for the ascorbate content on chromosomes 1 and 6 corresponded with QTLs for photosynthesis-related traits supports this conclusion. Nevertheless, the ascorbate content also seems to be determined by other genomic regions, which are not involved in the chilling tolerance of photosynthesis, as indicated by the QTL for the ascorbate content on chromosome 8. This may explain the unstable correlations between the ascorbate content and the chilling tolerance of the studied maize genotypes (e.g. Hodges et al., 1996; Leipner et al., 1999a).

Similar to our previous studies (Fracheboud et al., 2004; Jompuk et al., 2005), the centromeric region of chromosome 2 harbored a major QTL for chlorophyll content. As well as the candidate genes discussed by Jompuk et al. (2005), we identified another potential candidate gene for this QTL, namely luteus18, an unknown gene, the mutation of which results in yellow seedlings. As mentioned above, the telomeric region of the long arm of chromosome 6 harbors the major QTL for chilling tolerance of photosynthesis (Fracheboud et al., 2004; Jompuk et al., 2005). Similar to the earlier studies, in which leaf greenness was not associated with this QTL, there was no association of this chromosomal region with the chlorophyll content. However, a QTL for the chlorophyll a/b ratio was identified at the position of the major QTL for chilling tolerance of photosynthesis. This QTL was characterized by positive additivity, meaning that the allele of ETH-DH7 was responsible for a higher chlorophyll a/b ratio. Similarly, the major QTL for chilling tolerance of photosynthesis in the Ac7643 × Ac7729 mapping population was also associated with a QTL for the chlorophyll a/b ratio, both with positive additivity (Fracheboud et al., 2002). Together with the finding that growth at suboptimal temperature induces a reduction in the chlorophyll a/b ratio, especially in chilling-sensitive maize genotypes (Haldimann, 1998), this indicates that a high chlorophyll a/b ratio is typical of maize seedlings with a high tolerance of photosynthesis to chilling. This raises the question as to whether the increase in the chlorophyll a/b ratio is due to a mechanism, which actively induces higher chilling tolerance, or due to a pleiotropic effect of underlying genes. Furthermore, it raises the question of the molecular cause of the altered chlorophyll a/b ratio. Since photosystem I (PSI) is characterized by a high chlorophyll a/b ratio (Thayer and Björkman, 1992), the chilling tolerance allele of ETH-DH7 may be responsible for a higher level of PSI. On the other hand, since the light-harvesting complexes contain chlorophyll a and b, while the reaction centre core complex contains only chlorophyll a (Peter and Thornber, 1991), the ETH-DH7 allele would result in a lower ratio of the light-harvesting complex to the core complex. Both mechanisms would result in a lower excitation pressure towards PSII. The greater amount of PSI would induce better electron drainage from PSII and the lower ratio of the light-harvesting complex to the core complex would directly reduce the excitation pressure from the light-harvesting complex towards the core complex. For both mechanisms, potential candidate genes, which were found to be expressed upon exposure to cold stress (Singh et al., 2001), are located near this QTL. One of these genes is psaH, which codes for PSI-H, a subunit of PSI. The PSI-H seems to play an important role in the state transition as was found in mutants of Arabidopsis with low levels of psaH (Lunde et al., 2000). Another candidate gene is cab-m7, which codes for the LHC II proteins Lhcbm7. Cab-m7 is preferentially expressed in maize mesophyll cells upon illumination (Becker et al., 1992). Co-localization of Lhcb genes with QTLs for cold-induced photoinhibition was also reported in another study of maize QTLs (Pimentel et al., 2005). In conclusion, genotypic differences in the chilling tolerance of photosynthesis may be present without changes in the extractable activity of C4 cycle enzymes or in the content of antioxidants. The relationship between the chilling tolerance of photosynthesis and the activity of C4 cycle enzymes, respectively the content of antioxidants, which was frequently identified in earlier studies, might be coincidental; therefore, they seem not to be reliable markers of chilling
tolerance in maize seedlings. On the other hand, the chlorophyll a/b ratio seems to be associated with the chilling tolerance of photosynthesis. Whether this is an actual pleiotropic effect or not remains to be determined. Moreover, it is still unclear whether the change in the chlorophyll a/b ratio is the primary cause of chilling tolerance or a consequence of chilling tolerance.
Chapter 10

Genetic analysis of pleiotropic relationships between photosynthetic performance and specific leaf area of maize seedlings*

The objective of this study was to elucidate the genetic relationship between the specific leaf area (SLA) and the photosynthetic performance of maize (Zea mays L.) as dependent on growth temperature. Three sets of genotypes: (i) 19 S₅ inbred lines, divergently selected for high or low photosystem II operating efficiency ($F'_{q'/F_{m'}}$) at low temperature, (ii) a population of 226 F₂:₃ families from the cross of ETH-DL3 × ETH-DH7 and (iii) a population of 168 F₂:₄ families from the cross of Lo964 × Lo1016 were tested at low (15/13 °C day/night) or at optimal (25/22 °C day/night) temperature. The latter cross was originally developed to study QTLs for root traits. At 15/13 °C the groups of S₅ inbred lines selected for high or low $F'_{q'/F_{m'}}$ differed significantly for all the measured traits, while at optimal temperature the groups differed only with regard to leaf greenness (SPAD). At low temperature, the SLA of these inbred lines was negatively correlated with $F'_{q'/F_{m'}}$ ($r = -0.56$, $p < 0.05$) and SPAD ($r = -0.80$, $p < 0.001$). This negative relationship was confirmed by mapping quantitative trait loci (QTL) in the two mapping populations. A co-location of three QTLs for SLA with QTLs for photosynthesis-related traits was detected in both populations at 15/13 °C, while co-location was not detected at 25/22 °C. The co-selection of SLA and $F'_{q'/F_{m'}}$ in the inbred lines and the co-location of QTL for SLA, SPAD and $F'_{q'/F_{m'}}$ at 15/13 °C in the QTL populations strongly support pleiotropy. There was no evidence that selecting for high $F'_{q'/F_{m'}}$ at low temperature leads to a constitutively altered SLA.

Introduction

In temperate regions, the maize crop is often exposed to long-term low temperature during early phases of development, resulting in poor photosynthetic performance (Stirling et al., 1991) and the maintenance of a high carbon exchange rate (CER) is among the most limiting factors for cold tolerance (Lee et al., 2002a). At optimal temperatures, when CER varies little among genotypes, other factors, like the rate of development might be of greater importance, leading to an earlier canopy closure and thus to an increased light interception (Richards, 2000). The necessary increase in leaf area can be achieved by a change in partitioning of carbohydrates between or within organs. For example, a high specific leaf area (SLA, ratio of leaf area to leaf weight) is one way by means of which barley achieves its early growth advantage over wheat (Lopez-Castaneda et al., 1995). However, there is evidence that the SLA is negatively correlated with the CER at various irradiances and concentrations of atmospheric CO₂ (Evans, 1998; Poorter and Van der Werf, 1998). Fichtner et al. (1993) reported an increase in the SLA in tobacco plants transformed with antisense rbcS (Rubisco small subunit), which were characterized by a lower CER and chlorophyll content due to the lower level of Rubisco. In maize, a negative relationship between SLA and CER was found for three tropical and three temperate inbred lines early sown in the field (Verheul et al., 1996). The above studies indicate that SLA is negatively correlated with CER whenever the variation in the CER, due to environmental or genetic influences, is large. In contrast to the negative relationship between SLA and photosynthetic performance, in general a high SLA and, thus, a high rate of CO₂ assimilation per unit leaf mass seems to be most important for achieving fast growth. For example, a high SLA is considered to be an important trait of crops like sugarcane (Terauchi and Matsuoka, 2000) and temperate cereals (Richards, 2000), leading to a higher light interception during early development. Furthermore, slow-growing alpine species have a lower SLA than fast-growing lowland species (Atkin and Lambers, 1998). In a comprehensive review based on 57 studies, Poorter and Van der Werf (1998) concluded that the leaf area ratio (LAR, ratio of leaf area to plant weight), and more specifically SLA, are the most important factors in the variation of the relative growth rate. The introduction of exotic maize germplasm, showing temperature stable, strong photosynthetic

* based on the following publication:
performance, in European breeding programs is considerably limited by its linkage to a small SLA (Leipner et al., 1999b; Soldati et al., 1999; Stehli et al., 1999). It has been shown that an improvement in the CER at low temperature can be achieved indirectly by selecting for a high photosystem II operating efficiency \((F_{q}/F_{m}'\)) at low temperature (Fracheboud et al., 1999). The chlorophyll fluorescence parameter \(F_{q}/F_{m}'\) measures the proportion of the light absorbed by chlorophyll associated with PSII that is used for photochemistry (Genty et al., 1989). At constant light intensity there is a strong linear correlation between \(F_{q}/F_{m}'\) and CER as it was shown, for example, in maize seedlings grown at suboptimal temperature (Fracheboud et al., 2004). Furthermore \(F_{q}/F_{m}'\) measurements are much faster compared to CER measurements taking only few seconds in comparison to several minutes. This makes \(F_{q}/F_{m}'\) a useful tool for the routine screening of the photosynthetic performance of maize under suboptimal growth temperatures. However, since maize is not constantly exposed to suboptimal temperatures during its life cycle, a constitutive high photosynthetic capacity, if achieved in combination with a small SLA, is an undesirable attribute (Leipner et al., 1999b).

The research herein presented was conducted in maize in order to: (i) determine whether a divergent selection for \(F_{q}/F_{m}'\) at low temperature led to a co-selection of SLA and greenness, (ii) evaluate whether this selection led to a constitutive expression of low SLA and (iii) verify whether QTLs for SLA were co-located with QLTs for other traits in two mapping populations.

**Materials and methods**

**Plant material**

The plant material consisted of a set of 19 experimental inbred lines and two mapping populations of maize. The inbred lines had been divergently selected for the photosystem II operating efficiency \((F_{q}/F_{m}'\)) at low temperature \((15/13 \, ^{\circ}C\), day/night\). The selection was from three breeding populations (i.e. one dent, one flint and one exotic population with a 25% flint and 75% Mexican highland background; Chapter 3; Fracheboud et al., 1999). After four cycles of inbreeding selection, the families were further selfed to obtain S\(_{5}\) inbred lines. For this study, 11 of the lines selected for high photosynthetic efficiency \((H)\) and eight selected for low photosynthetic efficiency \((L)\) were available: seven dent lines \((D)\), four exotic lines \((E)\) and eight flint lines \((F)\): ETH-DH1 \((a)\), ETH-DH2 \((b)\), ETH-DH5 \((c)\), ETH-DH7 \((d)\), ETH-DL3 \((e)\), ETH-DL4 \((f)\), ETH-DL7 \((g)\), ETH-EH2 \((h)\), ETH-EH3 \((i)\), ETH-EH5 \((k)\), ETH-EL2 \((l)\), ETH-FH1 \((m)\), ETH-FH6 \((n)\), ETH-FH7 \((o)\), ETH-FH8 \((p)\), ETH-FL1 \((q)\), ETH-FL3 \((r)\), ETH-FL5 \((s)\), ETH-FL8 \((t)\). The letters in brackets identify the inbred lines in Figure 1.

From the cross of ETH-DL3 × ETH-DH7 a QTL population of 226 F\(_{2:3}\) families was developed (Chapter 4; Fracheboud et al., 2004), referred to as ETH-population. The second population, referred to as Lo-population, consisted of 168 F\(_{2:4}\) families derived from the Lo\(_{964}\) × Lo1016 cross (Tuberosa et al., 2002), two dent lines contrasting in root morphology (Sanguineti et al., 1998) and the cold tolerance at germination (Frascaroli et al., 2005). The parents and the F\(_{2:3}\) families of the Lo-population were provided by Dr. M. Motto (Experimental Institute for Cereal Crops, Bergamo, Italy). The F\(_{2:4}\) seeds were produced at the Department of Agro-environmental Science and Technology (DISTA, Bologna, Italy).

**Growth conditions**

The set of 19 experimental inbred lines and the ETH-population were grown in growth chambers \((PGW36,\) Conviron, Winnipeg, Canada) in 1-l pots containing a commercial mixture of soil, peat and compost \((Torf und Pikiererde 140,\) Ricoter, Aarberg, Switzerland). Two treatments were applied, i.e. optimal and suboptimal temperatures. The control plants grew for 13 days at 25/22 °C (optimal temperature) with a photoperiod of 12 h at 400 µmol m\(^{-2}\) s\(^{-1}\) and a relative humidity of 60/70% (day/night). Chilling-treated (suboptimal temperature) plants were first grown for 6 days as control plants and then for 14 days at 15/13 °C; the other conditions were the same as those for the control plants. In both temperature regimes the plants were measured and harvested at the 3-leaf \((V3)\) stage, i.e. when the whole collar of the third leaf was visible.

For the Lo-population, pre-germinated seeds were placed in growth columns \((7 \, \text{cm diameter,} \, 25 \, \text{cm height})\) in a mixture of quartz sand \((\text{particle size } 0.08–0.2 \, \text{mm})\) and 5% \((w/w)\) vermiculite powder \((Vermex Pulver E,\) Vermica AG, Bözten, Switzerland). The sand substrate was used to ease the evaluation of root traits after harvest, the results of which are described elsewhere (Hund et al., 2004). After coleoptile emergence, the plants were grown under the same conditions as the plants at suboptimal temperature treatment described above. The plants of the Lo-population were harvested after 21 days when most had reached the 1-leaf \((V1)\) stage.

**Determination of morpho-physiological traits**

All the measurements on the ETH material \((S_{5} \, \text{lines and the ETH-population})\) were performed on fully expanded third leaves after 14 \((25/22 \, ^{\circ}C)\) and 20 \((15/13 \, ^{\circ}C)\) days. The photosystem II operating efficiency \((F_{q}/F_{m}'\)) and the CER were measured as described by Fracheboud et al. (2004) with a LI-6400 instrument equipped with an LI-6400–40 pulse-
amplitude modulation fluorometer (LI-COR, Lincoln, NE, USA). The greenness of the third leaf was recorded with a chlorophyll meter (SPAD-502, Minolta Corporations, Ramsey, NJ, USA). The area of the third leaf was measured with a leaf-area meter (LI-COR 3100, Lincoln, NE, USA); its dry weight was recorded after drying at 65 °C for 42 h. The SLA was calculated as SLA = leaf area/leaf dry weight (m² kg⁻¹).

For the Lo-population, all the morpho-physiological measurements were performed on the second leaf after 19 and 20 days (blocks 1 and 2, respectively) after imbibition. The F₄'/Fₘ' was measured at 400 µmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD) with a pulse-amplitude modulated fluorometer (PAM-2000, Walz, Effeltrich, Germany) as described by Hund et al. (2004). The SPAD values of the second leaves and the leaf area of the fully developed first leaf and the successive leaves were measured as described above. SLA was calculated as the overall leaf area per overall leaf dry matter.

**Experimental design and statistics**

For the evaluation of the experimental inbred lines, a randomized complete-block design combined over temperature treatments was used. Each temperature treatment consisted of one growth chamber run containing three replications and each experimental unit consisted of one pot containing two plants. The plants of the ETH-population were sown and analyzed at intervals of 24 h in incomplete blocks of 22 families. Six to nine plants of each F₂;₃ family and six plants of each parental line were analyzed at each growth temperature (for further details see Chapter 4 and Fracheboud et al., 2004).

The plants of the Lo-population, including the two parental inbred lines and the 168 F₂;₄ families, were arranged in a randomized complete-block design with two growth chamber runs and two replications per run. For technical reasons different growth chambers were used, slightly differing from each other (50 µmol m⁻² s⁻¹ PPFD and about 0.5 °C) and were thus considered as different environments. Experimental units (PVC columns), each containing three plants, were arranged as a central and a border block, according to the light intensity in the growth chamber. All the data were analyzed using the general linear model procedure (PROC GLM) of SAS 8.02 (SAS Institute Inc., 1999–2001, Cary, NC, USA). Skewness was calculated with the e1071 package in R (Ihaka and Gentleman, 1996).

**Quantitative trait loci analysis**

QTLs were identified by composite interval mapping using QTL Cartographer (Jansen and Stam, 1994; Zeng, 1994) with different model thresholds, depending on the population and the experimental design. The QTL analyses of the ETH-population were performed with a linkage map with 118 SSR markers (Fracheboud et al., 2004). The QTLs were identified using model 6 of QTL Cartographer, with a blocking window size of 30 cM. The cofactors were selected by forward and backward regressions with the ‘in’ and ‘out’ thresholds at a p-value of 0.01. The presence of a QTL was considered to be significant when the likelihood of odds (LOD) value was higher than 3.5. This value corresponds to an experiment wise type I error rate (α') of 0.021 for a single trait analysis in an F₂ population.

**Figure 1**: Relationship between chlorophyll content (SPAD) and SLA and PSII operating efficiency (F₄'/Fₘ') and SLA of inbred lines selected for high (circles) and low (triangles) F₄'/Fₘ' at low temperature. Lines were grown at 15/13 °C (closed symbols) or 25/22 °C (open symbols). See Material and methods with regard to letters, which indicate the line. Bars (left hand corner) indicate Fisher’s least significant difference (LSD) for the comparison of trait values at optimal (H) and suboptimal (L) growth temperature.
population with three degrees of freedom (df), assuming that all the chromosomes segregate independently.

The QTL analyses of the Lo-population were performed with a linkage map with 135 markers (Tuberosa et al., 2002). The co-factors were selected as described by Hund et al. (2004). A QTL was declared significant when a critical LOD threshold of 3.5 was exceeded. For a joint analysis of an F2 population in two environments (i.e. growth chambers) this threshold is equivalent to α’ of 0.11. A joint analysis of the phenotypic data of the two growth chamber runs enabled the evaluation of the QTL-by-environment (Q×E) interaction (Jiang and Zeng, 1995). The factor ‘environment’ accounts for the variability in time and space. An LOD threshold of 1.3 for a significant Q×E interaction was based on the type-I error rate of 0.05 for a single locus for an F2 with two df. Multiple regressions were used to evaluate the actual additive effects of the QTL and the total percentage of phenotypic variation accounted for by the identified QTL. Considering that the phenotypic evaluation was made on the F2:4, only the additive effects of the QTLs are reported.

### Results

For the experimental inbred lines, the analysis of variance revealed significant differences between temperature treatments, genotypes and their interaction (p < 0.001) for all evaluated traits. A separate analysis of the optimal and suboptimal temperature treatments showed highly significant differences among the genotypes (p < 0.001) for all traits at both temperatures (data not shown). At suboptimal temperature significant differences were detected between the group of genotypes selected for high \( F_q/F_m' \) (H) and the group selected for low \( F_q/F_m' \) (L) for all the measured traits (Table 1). At optimal temperature, the only significant difference between the two groups was in the SPAD values. At suboptimal temperature, there were modest to very strong correlations among the measured traits (Table 2). The SLA correlated negatively with all the other traits; the strongest correlation was with SPAD values. At suboptimal temperature, a very strong correlation was found between CER and \( F_q/F_m' \). At optimal temperatures, there were no significant correlations among the traits, with the exception of a strong correlation between CER and \( F_q/F_m' \). The significant correlations between values of SLA and SPAD and of SLA and \( F_q/F_m' \) for inbred lines selected for high and low \( F_q/F_m' \) at low temperature are given in Figure 1. The two groups of inbred lines are displayed with different symbols in order to underline that for these materials, the high correlation is not only due to differences among groups, but also within groups.

With respect to the ETH-population, the growing temperature had a significant effect on all traits. The values of SLA, SPAD, \( F_q/F_m' \), CER and shoot dry weight at 25 °C were 52.5 m² kg⁻¹, 37.2, 0.52, 15.0 µmol m⁻² s⁻¹ and 218 mg and where significantly reduced at low temperature by 13.5, 45.2, 74.6, 81.7 and 25.2%, respectively. The variation among F2:3 families was significant at both temperatures. The correlations between SPAD values or \( F_q/F_m' \) with SLA at low temperatures were not as close as expected from the results obtained from the S5 inbred lines. The phenotypic correlation coefficients between SLA and the other above traits ranged from -0.23 (CER) to -0.38 (shoot dry weight) for plants grown at 15 °C and from -0.15 (shoot dry weight) to -0.28 (SPAD) for plants grown at 25 °C (data not shown). The QTL analysis of the ETH-population grown at 25 °C revealed two significant QTLs for SLA, whereas at 15 °C a total of four QTLs for SLA were detected (Table 3). There were no common QTLs for the 15 °C-

### Table 1: Mean values of SLA (m² kg⁻¹), leaf greenness (SPAD), PSII operating efficiency (\( F_q/F_m' \)), and CER (µmol CO₂ m⁻² s⁻¹) of 19 S5 inbred lines selected for high (H) and low (L) \( F_q/F_m' \) at low temperature.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Selection</th>
<th>SLA</th>
<th>( F_q/F_m' )</th>
<th>CER</th>
</tr>
</thead>
<tbody>
<tr>
<td>15/13 °C</td>
<td>H</td>
<td>57.6</td>
<td>0.25</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>66.5</td>
<td>0.10</td>
<td>3.0</td>
</tr>
<tr>
<td>Significance</td>
<td></td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>25/22 °C</td>
<td>H</td>
<td>50.8</td>
<td>0.50</td>
<td>16.8</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>51.2</td>
<td>0.49</td>
<td>17.4</td>
</tr>
<tr>
<td>Significance</td>
<td></td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

***Significance for the contrast between the ‘H’ and ‘L’ group at p ≤ 0.001; ns: not significant.

### Table 2: Pearson correlation coefficients between mean values of photosynthesis-related traits of 19 S5 inbred lines divergently selected for \( F_q/F_m' \) at low temperatures.

<table>
<thead>
<tr>
<th>15 °C</th>
<th>CER</th>
<th>( F_q/F_m' )</th>
<th>SPAD</th>
<th>SLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CER</td>
<td>0.98***</td>
<td>0.78***</td>
<td>-0.63***</td>
<td></td>
</tr>
<tr>
<td>( F_q/F_m' )</td>
<td>0.83***</td>
<td>0.73***</td>
<td>-0.58***</td>
<td></td>
</tr>
<tr>
<td>SPAD</td>
<td>-0.03</td>
<td>0.07</td>
<td>-0.80***</td>
<td></td>
</tr>
<tr>
<td>SLA</td>
<td>-0.27</td>
<td>-0.04</td>
<td>0.07</td>
<td></td>
</tr>
</tbody>
</table>

Correlations at 15/13 °C (day/night) (above the diagonal), and 25/22 °C (below the diagonal). Significance of the regressions at p ≤ 0.05, p ≤ 0.01, and p ≤ 0.001 is indicated by *, **, and ***, respectively. The scatter plots corresponding to the correlation coefficients (bold) are shown in Figure 1.
with QTLs for SLA are shown. The map and the QTLs for filled bars indicate QTLs of plants developed at suboptimal temperature (15 °C). Bars to the left of the chromosome indicate QTLs of plants grown at optimal temperature (25 °C), whose parental inbred lines were not selected for photosynthetic traits in the cold, was used to test the association between $F_q/F_m'$, SPAD and SLA in an independent set of genotypes. According to the analysis of variance the two growth chamber runs were significantly different for all three traits (data not shown). The parents differed significantly ($p < 0.001$) in $F_q/F_m'$ and SPAD. All frequencies were more or less normally distributed, but the direction of the asymmetric tail (skewness) depended on the growth chamber run. The skewness of the distribution of $F_q/F_m'$ and SPAD was negative in replication 1 (-0.52 and -0.53, respectively) and positive in replication 2 (0.57 and 0.10, respectively). For the $F_{2:4}$ families of the Lo-population there was a significant genotype-by-environment (growth chambers) interaction for $F_q/F_m'$ and SPAD but not for SLA. The correlations between $F_q/F_m'$, SPAD and SLA were closer in the second growth chamber run, in which the variability of all trait values was higher (Figure 3). The QTL analysis revealed three QTLs for SLA, which were located on chromosomes 1, 5 and 10 (Table 4). In replication 1, the locus on chromosome 10 ex-

### Table 3: Putative QTLs for the SLA (m² kg⁻¹) (LOD > 3.5) of 226 $F_{2:4}$ families of the ETH-population (ETH-DL3 × ETH-DH7) grown at suboptimal (15/13 °C) or optimal (25/22 °C) temperature.

<table>
<thead>
<tr>
<th>Temp</th>
<th>Chr</th>
<th>cm</th>
<th>Nearest marker</th>
<th>LOD</th>
<th>$R^2$</th>
<th>Add *</th>
<th>Dom</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 °C</td>
<td>1</td>
<td>209</td>
<td>bnlg1502</td>
<td>4.1</td>
<td>0.06</td>
<td>-0.97</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>108</td>
<td>phi109964</td>
<td>5.1</td>
<td>0.09</td>
<td>-1.18</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>221</td>
<td>bnlg1740</td>
<td>6.6</td>
<td>0.12</td>
<td>-1.13</td>
<td>-1.84</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>132</td>
<td>bnlg1028</td>
<td>4.7</td>
<td>0.08</td>
<td>0.05</td>
<td>1.26</td>
</tr>
<tr>
<td>25 °C</td>
<td>5</td>
<td>123</td>
<td>bnlg386</td>
<td>5.6</td>
<td>0.09</td>
<td>0.85</td>
<td>-0.64</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>78</td>
<td>bnlg1031</td>
<td>5.6</td>
<td>0.09</td>
<td>-0.95</td>
<td>0.55</td>
</tr>
</tbody>
</table>

* Additive and dominant effects represent the substitution of an ETH-DL3 allele with an ETH-DH7 allele.

Figure 2: QTLs for SLA, leaf greenness (SPAD), shoot dry weight (DW), CER, and PSII operating efficiency ($F_q/F_m'$) above an LOD threshold of 3.5 in the ETH-population. Hatched bars indicate QTLs of plants grown at optimal temperature (25 °C), filled bars indicate QTLs of plants developed at suboptimal temperature (15 °C). Bars to the left of the chromosome represent QTLs with negative additivity, bars to the right of the chromosome QTLs with positive additivity. Only, chromosomes with QTLs for SLA are shown. The map and the QTLs for $F_q/F_m'$, CER, SPAD, and shoot dry weight were taken from Fracheboud et al. (2004) (see also Chapter 4).
plained the highest portion of variability for SLA, while in replication 2 this was the case for the loci on chromosomes 1 and 5. By comparing with previous results (Hund et al., 2004), for all the SLA QTLs, a co-location with QTLs for leaf greenness was observed (Figure 4). Moreover, a QTL for $F'_q/F'_m$ (chromosome 1) and QTLs for $F'_q/F'_m$ and shoot dry weight (chromosome 10) were located at the same positions as the QTLs for SLA. The additivity of the QTLs for SLA always had the opposite sign to those for the other traits. There was no overlap of the QTLs for SLA between the Lo and the ETH populations.

Discussion

Improving photosynthesis at low temperature is one of the most relevant breeding goals for the adaptation of maize to low temperature during early growth in a temperate climate. In the present work, we used inbred lines, which were exclusively selected for high or low $F'_q/F'_m$ at low temperature (Chapter 3; Fracheboud et al., 1999) to study the co-selection of other traits. Divergent selection was successful and led to an increase in the CER, of up to 31%, in hybrids derived from inbred lines selected upwards (Fracheboud, 1999). In contrast to studies with unrelated, contrasting genotypes, the material used has the advantage that the association between traits is not biased by selection pressure on traits other than $F'_q/F'_m$. Grown at suboptimal temperature, the group of lines selected for high $F'_q/F'_m$ differed significantly from the group selected for low $F'_q/F'_m$ for all the traits. This supports the hypothesis that pleiotropic effects are the cause of the close phenotypic correlations among these traits at suboptimal temperature. At optimal temperature, significant effects between the two groups were found only for leaf greenness. Thus, the selection did not constitutively change the leaf physiology and morphology. This is important since an unfavorable linkage between temperature stable high $F'_q/F'_m$ and low SLA might lead to a decrease in productivity at optimal temperature (Leipner et al., 1999b; Poorter and Van der Werf, 1998). The association between photosynthetic performance and SLA was observed in a previous study in maize (Verheul et al., 1996). However, unrelated genotypes had been used, which were pre-selected

<table>
<thead>
<tr>
<th>Chr</th>
<th>cM</th>
<th>Nearest marker</th>
<th>LOD Joint</th>
<th>Q×E</th>
<th>Repl. 1</th>
<th>Repl. 2</th>
<th>$R^2$</th>
<th>Add*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>PGAMCTA310</td>
<td>5.0</td>
<td>0.6</td>
<td>0.07</td>
<td>0.09</td>
<td>-1.88</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>118</td>
<td>PGAMCGT165</td>
<td>4.4</td>
<td>2.8</td>
<td>0.03</td>
<td>0.08</td>
<td>-1.31</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>59</td>
<td>PGAMCTT250</td>
<td>4.0</td>
<td>0.8</td>
<td>0.13</td>
<td>0.02</td>
<td>1.95</td>
<td></td>
</tr>
</tbody>
</table>

Chr, chromosome number; cM, position of the peak of the QTL in centimorgan; Joint, joint analysis of the two experiments; Q×E, QTL-by-environment interaction; Repl., replication; $R^2$, phenotypic variance explained by genotype class at LOD peak; Add, additivity. * Additive effects represent the substitution of a Lo964 allele with a Lo1016 allele.

Figure 3: Relationship between chlorophyll content (SPAD values) and SLA and between the photosystem II operating efficiency ($F'_q/F'_m$) and SLA in the Lo-Population. The lines were grown at low 15/13 °C temperatures in two growth chamber runs (open and closed symbols). Circles: $F_{2.4}$ families; triangles: Lo1016; squares: Lo964. Error bars show 95% confidence intervals.

Table 4: The putative QTLs for SLA (LOD > 3.5) of 168 $F_{2.4}$ families of the Lo-population (Lo964 × Lo1016) grown at 15 °C.
for high and low vigor, as defined by a large leaf area and high greenness. Thus, the relationship between the traits may have been due to a co-selection of independent loci or to random genetic drift. Despite the low correlation between SLA and photosynthesis-related traits in the ETH-population, the QTL analysis of plants grown at suboptimal temperature yielded three of four QTLs where SLA was co-located with photosynthesis related traits. The opposite sign of the additive effects matches with the phenotypic observations. In contrast, the QTLs for SLA from seedlings developed at optimal temperature were not associated with QTLs for SLA at suboptimal temperature as well as with QTLs for other measured traits. Furthermore, a high SLA at optimal temperature had no positive effect on dry matter accumulation but was negatively correlated with the shoot dry weight at suboptimal temperature. This shows clearly that SLA was under different genetic control at suboptimal and at optimal temperature. The characteristics of leaves developed at suboptimal temperature compared to leaves grown at optimal temperature with respect to SLA, leaf greenness and photosynthetic performance are very similar to the characteristics of leaves grown at low and high light intensity (see for example Evans and Poorter, 2001). However, while in plants acclimated to low or to high light intensity the SLA was positively correlated with the assimilation rate per leaf weight (calculated from the data provided by Evans and Poorter, 2001), this was the case only when the ETH-population was grown at optimal but not at suboptimal temperature (data not shown). Therefore, it seems unlikely that the increase in the SLA of the chilling-sensitive genotypes at suboptimal temperature is an optimization reaction. Rather, it seems that a high SLA at suboptimal temperature is the consequence of a reduced availability of assimilates. The close (negative) relationship between SLA on the one hand and photosynthesis-related traits and shoot dry matter accumulation on the other was verified in the Lo-population as well as with QTLs for SLA at optimal temperature is the consequence of a reduced availability of assimilates.

The close (negative) relationship between SLA on the one hand and photosynthesis-related traits and shoot dry matter accumulation on the other was verified in the Lo-population as well as with QTLs for SLA at suboptimal temperature. The two different growth chamber runs were designed as replications in time. However, there was a significant difference between the two replications, which cannot be attributed to one distinct factor. The small difference of 50 µmol m$^{-2}$ s$^{-1}$ PPFD and of about 0.5 °C between the two growth chamber runs seem to cause the weaker performance of the plants in the second replication. However, the opposite skewness of the frequency distributions of the SPAD values and $F_\text{m}'/F_\text{m}$' in the two replications shows that the environmental conditions were in a range where the genotypes differed most. The significant genotype-
by-environment interaction for $F_q'/F_m'$ and SPAD was reflected by the significant QTL-by-environment interaction for many of the loci (see Hund et al., 2004). This indicates that, at the critical temperature threshold of 15 °C, small changes in the environmental conditions resulted not only in a shift in photosynthetic performance, but also in a shift in the set of genes responsible for its regulation. Despite these interactions, the association between $F_q'/F_m'$, leaf greenness and SLA was detected at the phenotypic as well as at the genetic level in both growth chamber runs.

By searching the maize genetics and genomics database, interesting candidate genes were identified for the major QTLs. For the ETH-population, promising candidate genes for the QTLs on chromosomes 2 and 6 were extensively discussed in the previous chapters. The major QTL of the Lo-population, which was localized at chromosome 1, is close to the gene cat2 when the Lo964 × Lo1016 map is aligned with the BNL2002 map (Maize Genetics and Genomics Database). The cat2 encodes catalase 2, which is expressed in photosynthetic tissue of maize (Scandalios et al., 1983). Catalase, an antioxidative enzyme that detoxifies hydrogen peroxide, was shown to be involved in the induced acclimation of maize seedlings to chilling stress (Prasad, 1997). The significance of catalase for photosynthesis was demonstrated in antisense tobacco plants lacking catalase; antisense suppression of catalase resulted in a strong decrease in $rbcS$ (Rubisco small subunit) expression and in a reduced photosynthetic capacity (Rizhsky et al., 2002). It is clear that the detected QTLs span large chromosome regions with possibly thousands of genes. Therefore getting the underlying gene out of the database would be highly unlikely. However, all the detected candidate genes ($ssu2$, $agp2$ and $cat2$) are indirectly associated with the functioning of the photosynthetic apparatus through CO2 fixation, storage and oxidative stress response, respectively. This matches observations of transgenic tobacco with reduced Rubisco levels and, thus, weaker photosynthesis and a higher SLA (Quick, 1998; Stitt and Schulze, 1994).

In conclusion, we can state that the finding of a relationship among $F_q'/F_m'$, leaf greenness and SLA at suboptimal temperature in two independent populations strongly supports pleiotropy more than linkage disequilibrium as the genetic basis of the relationship among traits. However, the question remains as to whether this increase in SLA, observed at low temperature with the photosynthesis decline, is due to a shortage of assimilates and the formation of leaf structure or to a compensation reaction. Our results did not provide evidence that selection for good photosynthetic performance at low temperature leads to a co-selection of a constitutive low SLA, which is not a desirable attribute because it can cause a low productivity. We therefore, consider $F_q'/F_m'$ to be a valuable trait for selecting genotypes with improved chilling tolerance, especially since the trait measurements are reasonably fast, as required for plant breeding applications. However, the actual merit of a selection for $F_q'/F_m'$ and its effects on SLA must be confirmed by testing plants under field conditions.
Chapter 11

Low temperature stress induces genes involved in photosynthesis and signal transduction in maize seedlings as studied by suppression subtractive hybridization*

Unfavorable environmental conditions such as cold induce the transcription of a range of genes in plants in order to acclimate to these growth conditions. To better understand the cold acclimation of maize (Zea mays L.) it is important to identify components of the cold stress response. For this purpose, cold-induced genes were analyzed using the PCR-select cDNA subtraction method. We identified several novel genes isolated from maize seedling exposed for 48 hours to 6 °C. Of 18 Zea mays cold-induced genes (ZmCOI genes) characterized, the majority share similarities with proteins with known function in signal transduction and photosynthesis regulation. RT-PCR was conducted for a selected group of genes, namely ZmCOI6.1, ZmAAC1, ZmDREB2A and ZmERF3, confirming the induction by low temperature. In addition, it was found that their expression was strongly induced by other abiotic stresses such as drought and high salt concentration, by stress signaling molecules such as jasmonic acid, salicylic acid and abscisic acid and by membrane rigidification. These results suggest that this group of genes is involved in a general response to abiotic stresses.

Introduction

Low temperature is one of the most important abiotic factors limiting growth, development and distribution of plants. Maize (Zea mays L.) originates from the subtropical regions and is known to be very sensitive to low growth temperature. The optimal growth temperature for maize lies between 30 and 35 °C (Miedema, 1982). Low temperature affects germination, seedling growth, early leaf development and overall maize crop growth and productivity (Miedema, 1982). In particular in temperate regions, maize is often exposed to low temperature during its early development resulting in poor photosynthetic performance (Leipner et al., 1999a; Stirling et al., 1991), which is associated with retarded plant development (Miedema, 1982).

On the cellular level, chilling can affect membranes and their lipid composition thereby changing the water status of the cells/plant (Gemel et al., 1989). However, the impact on the photosynthetic apparatus is considered to be especially important (Baker et al., 1994). Damage caused to developing or mature maize leaves by low temperature occurs primarily in the chloroplasts, leading to inhibition of photosynthesis and, consequently, to premature senescence (Foyer et al., 2002). Furthermore, it was found that leaves of maize, developed under cold conditions, are characterized by a lower photosynthetic capacity, lower quantum efficiencies of CO₂-fixation (ΦCO₂) and of electron transfer at PSII (Fq'/Fm' ) (Nie et al., 1992), by changes in the antioxidative defenses (Leipner et al., 1997), reductions in the carbon cycle activity (Kingston-Smith et al., 1997; Long, 1983) in comparison to leaves grown at more favorable temperatures. In addition to cold-specific damages, chilling may impair water absorption by the roots and water transport to the shoot, leading to drought stress (Aroca et al., 2001).

Acclimation to low temperature requires plants to sense the temperature decrease and to transmit this information by a signaling pathway to the nucleus (or the chloroplast) in order to induce the expression of genes important for acclimation (Browse and Xin, 2001). Signaling molecules such as the plant hormones salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA) and ethylene are important effectors of abiotic stress response. In maize, these molecules have been found to induce cold-acclimation resulting in a higher tolerance to low temperature (Janda et al., 1999; Janowiak et al., 2002; Lee et al., 1996). Hydrogen peroxide (H₂O₂) is regarded as another important signaling molecule and regulator of expression of some genes in response to stress (Foyer

* based on the following publication:
and Noctor, 2005). These include genes encoding proteins involved in the production of antioxidants, cell rescue/defense proteins and signaling proteins such as kinases and phosphatases and transcription factors (Vranová et al., 2002). Stress triggers the generation of ethylene that plays a role in executing cellular responses to the changed environment (Kim et al., 2003). Many cold-induced genes are also upregulated by other abiotic stresses often through the signaling molecules ABA and JA, suggesting that a common set of signal transduction pathways are employed during different stress responses (Shinozaki et al., 2003). The cross-talk between pathways could be explained by the presence of promoter sequence elements in the promoter region of stress induced genes that respond to several environmental stresses and signaling molecules.

Although much of knowledge in cold acclimation arises from Arabidopsis thaliana it is important to research directly in the cold sensitive crops to unravel its precise response pattern. Maize is sensitive to low temperature; however, it has the ability to acclimate to suboptimal temperature (about 14-20 °C) and, thus, to increase its tolerance to cold stress (Haldimann et al., 1996; Leipner et al., 1997). The response to low temperature is accompanied with changes in specific gene transcripts. The identity of some cold-induced genes in maize is known such as ZmDREB1A, ZmDBF1, ZmCDPK1, MLIP15, FAD7 and FAD8 (Berberich and Kusano, 1997; Berberich et al., 1998; Qin et al., 2004; Zheng et al., 2006). However, the exact function of these genes and encoded proteins in the cold response in maize is still not fully understood although it is known that some of their orthologues are important for the stress response in other plant species. Increased knowledge about the components of the maize stress response might present new strategies to improve maize through a higher stress tolerance.

In order to increase the understanding of cold acclimation in maize, a PCR-select cDNA subtraction method, also known as suppression subtractive hybridization (SSH), was selected to profile genes whose expression increases upon low temperature treatment. In comparison to other techniques, like for example DNA microarray technology, this method has the advantage that it allows to find novel genes. We identified a group of novel genes induced by cold stress where the majority of genes shared similarity on the amino acid level with known proteins in other plant species. In addition, further characterization showed that a selected group of genes was induced by abiotic stresses other then cold and by signaling molecules.

### Materials and methods

#### Plant material and growth conditions

Maize seeds of the genotype ETH-DH7 (Fracheboud et al., 2004) were grown in 1-L pots containing a commercial mixture of soil, peat and compost (Topf und Pikiererde 140, Ricoter, Aarberg, Switzerland). For treatment with signaling molecules seedlings were raised in half strength Hoagland solution (H2395, Sigma Chemical Co.) supplemented with 0.5% Fe-sequestrene, 6 mM K+ and 4 mM Ca2+. Plants were grown until the third leaf was fully developed at 25/22 °C (day/night) in growth chambers (Conviron PGW36, Winnipeg, Canada) at a 12-h photoperiod, a light intensity of 300 µmol m-2 s-1 and a relative humidity of 60%/70% (day/night).

#### Stress treatments and application of signaling molecules

Seedlings were exposed to stress conditions at the third-leaf stage. Control plants were harvested before application of the stress treatment. For the PCR-select cDNA subtraction method, cold stress was induced in seedlings by exposure to 6 °C for 48 h starting from the beginning of the photoperiod without changing light intensity, humidity and photoperiod. For the RT-PCR analysis, plants were kept in the dark during the application of all treatments to exclude an additional light-induced oxidative stress. Cold stress was induced by exposing the seedlings to 6 °C. To induce drought stress, maize plants were carefully removed from the soil and were left to dry in the growth chamber. The salt stress was induced by watering plants with 150 mM NaCl. Maize seedlings were placed in a growth chamber at 37 °C for the heat treatment. The third leaf was punctured several times along the whole leaf blade with a needle to cause mechanical wounding. The treatment with high light intensity was carried out by placing plants in the growth chamber at a light intensity of 1000 µmol m-2 s-1. Leaf samples were taken after 6 and 24 h. To induce changes in the membrane rigidity, dimethylsulfoxide (DMSO) was sprayed onto the leaves using a compressed air cylinder at concentrations of 35, 70, 140 or 280 mM. The plants were incubated for 24 h in the dark to exclude an additional light-induced oxidative stress. Control plants were sprayed with water.

Stress signaling molecules were added to the Hoagland solution to a final concentration of 30 µM jasmonic acid (JA), 100 µM salicylic acid (SA) and 100 µM abscisic acid (ABA). The H2O2 (10 mM) was sprayed onto the leaves. The incubation was performed in darkness and samples were taken after 6 and 24 h.
For all the treatments and controls, the middle part of the third leaf was sampled, then frozen in liquid nitrogen and stored at -80 °C until assay.

**Construction of the cDNA cold stress library**
Total RNA was isolated from the third leaf using TRIzol® according to Sigma's instructions for RNA isolation. The PCR-based cDNA subtraction was performed by using a PCR-Select cDNA Subtraction Kit (Clontech, Mountain View, CA, USA). "Tester" was the plant sample treated at 6 °C for 48 h and "driver" was the sample from plants grown at 25 °C. To obtain differentially expressed cDNAs, two rounds of hybridizations were performed, following the manufacturer's instructions of the PCR-Select cDNA Subtraction Kit. Afterwards, two rounds of PCR amplifications were performed for the subtracted cDNA. The subtracted cDNAs obtained from the second PCR amplification were cloned into pDrive vector (QIAGEN GmbH, Hilden, Germany). The transformed cells were plated onto LB agar culture plates containing ampicillin. A total of 2000 clones were selected from the LB plates and grown in liquid LB medium. Bacterial cultures were blotted onto Hybond blotting nylon membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was hybridized with double-stranded cDNA pools of equal specific activity derived from the subtracted or un-subtracted tester mRNA in DIG-Easy hybridization buffer for 15-18 h at 72 °C. Membranes were washed and then exposed to X-ray films. The signals of corresponding clones from two hybridizations were compared and the positive clones were selected. All the positive clones were sequenced by MWG (MWG-Biotech AG, Germany).

**Reverse transcriptase (RT)-PCR of detected cDNA sequences**
RT-PCR analysis was carried out to confirm differential expression of the detected sequences, which were found by the PCR-select cDNA subtraction method. First, total RNA was extracted from maize leaf samples using Tri Reagent® according to Sigma's protocol for RNA isolation. Then, total RNA of each sample was reverse transcribed to first-strand cDNAs using oligo (dT)23 primer according to the supplier's instructions (Advantage RT-for-PCR Kits, DB Biosciences, Clontech, Mountain View, CA, USA). The cDNA was amplified by PCR using the primers for ZmCOI6.1, ZmA 1 (ZmCOI6.10), ZmDREB2A (ZmCOI6.20) and ZmERF3 (ZmCOI6.21) (for primer sequences see Table 1). The amount of cDNA was semi-quantified by conducting only 25 PCR cycles. The maize gene coding for ubiquitin, ZmUBI (accession number S94466), was used as internal standard. Amplified PCR products were electrophoresed using 2.0% (w/v) agarose gel.

**Bioinformatics**
A similarity search was performed using the basic local alignment search tool (BLAST) at the National Centre for Biotechnology Information (NIH, Bethesda, MD, USA) (www.ncbi.nlm.nih.gov/ BLAST/). In particular, the blastn and blastx analyses were employed with an inclusion threshold (E-value) of 0.001.

### Table 1: List of oligonucleotides used in this work.

<table>
<thead>
<tr>
<th>ID</th>
<th>Primer sequence</th>
<th>Annealing temp (°C)</th>
</tr>
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<tbody>
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<td>ZmCOI6.1</td>
<td>F 5’-AGGTCGGGACAAAAAATGTTGC-3’</td>
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<tr>
<td>ZmA 1</td>
<td>F 5’-CGACGAGATGAGACCTA-3’</td>
<td>57</td>
</tr>
<tr>
<td>ZmDREB2A</td>
<td>F 5’-GTTAATTCATAGAGCCTGCGAG-3’</td>
<td>54</td>
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<td>ZmERF3</td>
<td>F 5’-CCCGCGGCTGGACAAGTCCC-3’</td>
<td>54</td>
</tr>
<tr>
<td>ZmUBI</td>
<td>F 5’-GGTGGTATGGAGACCTT-3’</td>
<td>57</td>
</tr>
<tr>
<td>Adapter 1</td>
<td>F 5’-CTAATACGACTCTATAACGCTCATGAGCGCCGCGCCGGCACGGG-3’</td>
<td>57</td>
</tr>
<tr>
<td>Adapter 2R</td>
<td>F 5’-CTAATACGACTCTACTATAGAGCGCCGCGCCGGCACGGG-3’</td>
<td>57</td>
</tr>
<tr>
<td>Nested PCR primer 1</td>
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</tr>
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<td>Nested PCR primer 2R</td>
<td>F 5’-TCCGGGCGGGCGGGCGGCAAGGT-3’</td>
<td>57</td>
</tr>
</tbody>
</table>
Results

Cloning and identification of differentially expressed cDNA

To identify novel cold-induced genes in maize that play a role in the cold stress response, seedlings of the chilling-tolerant genotype ETH-DH7 were exposed to 6 °C. The total RNA was extracted from the third leaf prior to treatment (control) and after 48 h exposure to 6 °C under a 12 h photoperiod. We used a 48 h time period in order to ensure induction and expression of genes which are important for the cold acclimation in maize.

The extracted RNA was analyzed with the PCR-select cDNA subtraction method to identify genes whose expression was induced or increased upon exposure to low temperature. The cDNA from stressed and control plants amplified by the PCR-select cDNA subtraction method, was cloned and screened for induction. Each clone was spotted onto two identical nylon membranes and hybridized with tester cDNA probe from plants exposed to 6 °C for 48 h and with control cDNA from plant grown at 25 °C, respectively. From the 2000 screened clones, a total of 69 candidate clones were classified as strongly cold-inducible and which produced a considerably stronger signal when probed with cDNAs derived from the cold-treated plants as compared to control cDNA. These clones were sequenced and the sequence data have been deposited at the GenBank database under the accession numbers DQ060243, DQ078760 to DQ078778. For some sequences a high percentage of replications were identified resulting in 17 different cDNA sequences (Table 2).

The 18 defined individual sequences represented mostly novel not yet characterized genes in maize. The candidate genes were named ZmCOI6 (Zea mays cold induced at 6 °C) followed by a number. To unravel potential function, a similarity search was performed using the basic local alignment search tool (blastn and blastx) for identification of homologous/orthologous sequences using the deduced amino acid sequence of the maize EST AY106347 3'-UTR flanking region. The deduced amino acid sequence of this AY106347 EST was highly similar to the phosphoribulokinase of rice (Oryza sativa). ZmCOI6.16 DNA sequence showed 96 % identity with the maize EST AY108897 3'-UTR region. The deduced amino acid sequence of AY108897 contained a rubrerythrin motif and an ACSF (aerobic cyclase system, Fe-containing subunit) domain showing high similarity to the hydrogenase Mg-protoporphyrin IX monomethyl ester cyclase from Hordeum vulgare (83 % identity).

As a result of the analysis described above, genes were grouped into functional categories based on their putative function according to FunCat (Ruepp et al., 2004) (Table 2). The first group linked to metabolism and contained genes involved in photosynthesis; ZmCOI6.5 (ZmPRK), ZmCOI6.9 (ZmMe3), ZmCOI6.15 (ZmrbcL) and ZmCOI6.16 (Zmacsf). One gene product was involved in protein synthesis, ZmCOI6.13 (Zm23S rRNA) and one in the protein fate, ZmCOI6.4 (ZmPARP). Two gene products belonged to the functional category "Cellular transport, transport facilities and transport routes"; they were the potential ADP-riboseylation factor (ZmCOI6.6, ZmARF) and the Ca2+-ATPase (ZmCOI6.10, ZmACA1). ZmCOI6.2 (ZmPRR) and ZmCOI6.14 (ZmASK3) were classified to the cellular communication and signal transduction mechanisms. To the functional category "Systemic interaction with the environment" belonged ZmCOI6.20 (ZmDREB2A) and ZmCOI6.21 (ZmERF3). Five gene products could not be classified.

Zea mays cold-induced genes are regulated by abiotic stresses, signaling molecules and membrane rigidification

Along the identified sequences, some of the candidate genes, namely ZmERF3 and ZmDREB2A, shared similarity to genes of transcription factors involved also in the response to other abiotic stresses
<table>
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<th>Annotation (Species)</th>
<th>GenBank accession</th>
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<th>Annotation (Species)</th>
<th>GenBank accession</th>
<th>E value</th>
<th>Identity</th>
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<td>a Phosphoribulokinase precursor (O. sativa)</td>
<td>BAD07865</td>
<td>~0</td>
<td>91%</td>
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<tr>
<td>ZmCOB9.9 (ZmMe3)</td>
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<td>ZmCOB4.4 (ZmARP)</td>
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<td>444</td>
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<td>Putative selenium binding protein (OsSBP), mRNA (O. sativa)</td>
<td>AB059401</td>
<td>4·e-68</td>
<td>86%</td>
<td>Putative selenium binding protein (O. sativa)</td>
<td>BAB6133</td>
<td>2·e-41</td>
<td>83%</td>
<td></td>
</tr>
<tr>
<td>ZmCOB1.3 (ZmHRGP)</td>
<td>1344</td>
<td>DQ060243</td>
<td>CL3471_1, mRNA sequence (Z. mays)</td>
<td>AY110815</td>
<td>~0</td>
<td>97%</td>
<td>Hypothetical protein Os1_010439 (O. sativa)</td>
<td>EAY89206</td>
<td>6·e-48</td>
<td>42%</td>
<td></td>
</tr>
</tbody>
</table>

^a^ = versus similarity to PC0092382, ^b^ = versus similarity to PC0080076.
the transcript levels of Cold, drought and salt treatment strongly increased the transcript level existed between experiments. while for ZmDREB2A point 12 h). An exception was increase (time point 6 h) and then to decrease (time these stresses, the transcript level seemed first to increase after treatment (6 and 24 h) were included. The selected genes were analyzed under drought, salt stress, high light intensity (HL), heat shock and mechanical wounding. Under non-stressed conditions, the levels of ZmCOI6.1 and ZmACA1 were hardly detectable while for ZmDREB2A and ZmERF3 some variation of the transcript level existed between experiments. Cold, drought and salt treatment strongly increased the transcript levels of ZmCOI6.1, ZmACA1, ZmDREB2A and ZmERF3. These transcripts were distinctly induced in maize leaves 6 h after treatment with cold, drought and salt treatment and their abundance was at least at the same level also 24 h after treatment. Heat shock, high light intensity and mechanical wounding did not increase expression as did the other stress treatments (Figure 1). Under these stresses, the transcript level seemed first to increase (time point 6 h) and then to decrease (time point 12 h). An exception was ZmCOI6.1 with which the transcript level hardly changed between 6 and 12 h.

To examine if the expression of the selected ZmCOI genes is regulated downstream of stress signaling molecules, the maize seedlings grown in hydroponics were exposed to the signaling molecules jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA) as well as to hydrogen peroxide (H$_2$O$_2$). Samples were collected from the third leaf at 6 and 24 h after the start of exposure of the roots to the signaling molecules. From the studied ZmCOI genes, ZmCOI6.1 and ZmDREB2A responded clearly to all signaling molecules by an induction of their transcripts (Figure 2). While the transcript level of ZmCOI6.1 seemed to be higher at 24 h than at 6 h the one of ZmDREB2A was apparently slightly lower. The ZmACA1 showed a similar pattern to ZmCOI6.1 with the exception that it did not respond to H$_2$O$_2$. The ZmERF3 transcript level increased specifically upon ABA treatment but only very little after exposure to JA, SA and H$_2$O$_2$ for 24 h. Membrane changes were induced under non-stressed conditions by applying the membrane rigidifier dimethylsulfoxide (DMSO) which mimics the effect of

**Figure 1:** The expression of ZmCOI6.1, ZmACA1, ZmDREB2A and ZmERF3 is increased upon exposure to abiotic stress other than cold. Time courses of activation of ZmCOI transcripts, ZmCOI6.1, ZmACA1, ZmDREB2A and ZmERF3, in maize leaves exposed to cold (6 ºC), drought, high light intensity (HL), 37 ºC (heat), mechanical wounding (wounding) and NaCl. Transcript levels were analyzed by RT-PCR. Transcripts derived from control plants (samples taken prior to treatment) are indicated with C. Maize ubiquitin gene (ZmUBI) was used as an internal control.

(Fujimoto et al., 2000; Qin et al., 2007). Therefore, the expression of ZmERF3 and ZmDREB2A under various stress conditions was studied by semi-quantitative RT-PCR. Furthermore, ZmCOI6.1 and ZmACA1 were included in this analysis due to their high frequency in the library. The latter was included also due to its potential involvement in the Ca$^{2+}$ signaling. The RT-PCR was employed using the primers mentioned in Table 1. Samples from cold-treated plants exposed to 6 ºC in the dark for shorter time exposure (6 and 24 h) were included. The selected genes were analyzed under drought, salt stress, high light intensity (HL), heat shock and mechanical wounding. Under non-stressed conditions, the levels of ZmCOI6.1 and ZmACA1 were hardly detectable with the exception that it did not respond to H$_2$O$_2$. The ZmERF3 transcript level increased specifically upon ABA treatment but only very little after exposure to JA, SA and H$_2$O$_2$ for 24 h. Membrane changes were induced under non-stressed conditions by applying the membrane rigidifier dimethylsulfoxide (DMSO) which mimics the effect of

**Figure 2:** ZmCOI6.1, ZmACA1, ZmDREB2A and ZmERF3 expression is increased differentially by stress signalling molecules and H$_2$O$_2$. Time courses of activation of cold-induced transcripts in maize leaves treated with stress signalling molecules jasmonic acid (JA, 30 µM), salicylic acid (SA, 100 µM), abscisic acid (ABA, 100 µM) and with hydrogen peroxide (H$_2$O$_2$, 10 mM). Transcript levels were analyzed by RT-PCR. Transcripts derived from control plants (samples taken prior to treatment) are indicated with C. Maize ubiquitin gene (ZmUBI) was used as an internal control.
Cold-induced genes in maize

Figure 3: ZmCOI6.1, ZmAACA1, ZmDREB2A and ZmERF3 expression is increased differentially by the membrane rigidification DMSO. Plants were treated with DMSO of concentrations between 35 to 280 mM to leaves at 25 °C in the dark. Samples were taken 24 h after treatment and the transcript levels were analyzed by RT-PCR. Transcripts derived from control plants (samples taken prior to treatment) are indicated with C. Maize ubiquitin gene (ZmUBI) was used as an internal control.

cold rigidification of the membrane (Örvar et al., 2000). To test if the expression of the selected ZmCOI genes (ZmCOI6.1, ZmAACA1, ZmDREB2A and ZmERF3) responds to changes of the rigidity of the membrane, we examined maize plants treated with DMSO applied to the leaf tissue at concentrations of 35, 70, 140 and 280 mM. Samples were harvested at 24 h after treatment. Seedlings treated with DMSO showed an increase of some ZmCOI transcripts (Figure 3). The studied ZmCOI genes, with the exception of ZmERF3, were induced at a concentration of and higher than 35 mM DMSO.

Discussion

Using the PCR-select subtraction technique, 18 genes were identified and sequenced whose expression was strongly induced or increased in maize seedlings upon long-term cold treatment. For several of these genes orthologous sequences were found in different plant species such as rice, barley, Arabidopsis and millet, suggesting that these genes are conserved within the plant kingdom. The identified Zea mays cold induced (ZmCOI) genes could be grouped in six categories (Table 2) according to their putative function. These genes were linked to photosynthesis, to protein synthesis and fate, to cellular transport facilities and signal transduction mechanisms as well as to systemic interaction with the environment. Furthermore, two novel genes coding for proteins with unknown function were detected. The diverse function of the cold induced genes found in this study is an indication of the complexity and the amount of different pathways involved in cold stress response in maize as shown also for other plants (Knight and Knight, 2001). Several genes among the ZmCOI genes were related to photosynthesis, underlining the importance of photosynthesis in the context of cold tolerance as it is commonly accepted from physiological studies (Baker et al., 1994). The expression of Rubisco and malic enzyme as well as of phosphoribulokinase induced by cold stress indicates that maize does not only try to minimize cold-induced photooxidative stress by promoting dissipative and antioxidative mechanisms (Foyer et al., 2002; Leipner and Stamp, 2009); seemingly, it also reduces the excitation pressure by increasing the sink capacity during acclimation to low temperature. The fact that these three enzymes were induced by low temperature may indicate that they are limiting factors for photosynthesis during cold stress. In C₄ plants, it was found that control of the photosynthetic flux seems to be shared by Rubisco and pyruvate orthophosphate dikinase (PPDK) (Sage and Kubien, 2007). In respect to malic enzyme, there is no evidence that this enzyme is rate-limiting during cold stress (Long, 1983). The role of phosphoribulokinase (PRK) during cold stress is largely unknown. Since PRK is redox, respectively light, regulated (Schürmann and Buchanan, 2001), it is very likely that its activity is strongly affected by cold induced photooxidative stress. There was, however, only limited accordance with similar studies using different maize genotypes and cold stress conditions as well as other approaches for gene identification. Two ZmCOI genes, namely ZmOPR1 and ZmME3, were also identified as cold induced genes in maize leaves using a drought stress-specific cDNA macroarray (Zheng et al., 2006). Moreover, expressed sequence tags with very high similarity (E-value < 1·e-100) to ZmARF, ZmPRR and ZmOPR1 were found in maize seedlings exposed for 4 days to 10 °C at 800 µmol m⁻² s⁻¹ as well as an EST highly similar to ZmASK3 when seedlings were gradually cooled to 5 °C at 125 µmol m⁻² s⁻¹ (Singh et al., 2001). The observation that only a limited number of genes were identical in these studies points to a flexible or redundant response of maize to cold stress. However, certain key-players seem to exist whose expression is always induced when maize plants are exposed to cold stress. Such a flexibility of a biological system has been discussed as a major requirement for a high robustness and, therefore, for a high tolerance against stress in biological systems (Stelling et al., 2004).

It has been suggested that cold signaling in plants may proceed from membrane rigidification through a re-organization of actin microfilaments to Ca²⁺ influx and to the expression cold acclimation genes (Örvar et al., 2000). The transcription of these cold-induced genes might be also regulated by various transcription factors (Shinozaki et al., 2003). Therefore, we
selected for a more detailed study by semiquantitative RT-PCR three of the ZmCOI genes, which are involved in Ca$^{2+}$ signaling (ZmACA1) and the regulation of transcription (ZmDREB2A and ZmERF3). Furthermore, the gene ZmCOI6.1 was chosen due to its high frequency in the subtracted cDNA library.

The ZmACA1 gene, which codes for a protein very similar to plasma membrane P-type Ca$^{2+}$-ATPase, was induced by different stresses, corroborating the observation that a wide array of stresses is accompanied by transient changes in the concentration of cytosolic free calcium (Knight and Knight, 2001). The observed induction of the ZmACA1 transcript in this study indicates that a regulation of P-type Ca$^{2+}$-ATPase occurs at the transcriptional level, as was also suggested for the stomatal guard cells (Schiøtt and Palmgren, 2005), besides the well-documented regulation on the post-transcriptional level (Dietz et al., 2001).

In maize, the ZmDREB2A gene was transcriptionally activated by cold, similarly to the DREB1/CFB members (Nakashima and Yamaguchi-Shinozaki, 2006). The DREB2 of rice and Arabidopsis is known to respond to drought and salt but not to cold (Dubouzet et al., 2003; Liu et al., 1998). Similarly to the situation in maize, the expression of a DREB2 homologue in wheat was found to be activated also by cold despite its induction by drought and salt stress (Egawa et al., 2006). In contrast to the CBF/DREB transcription factors, the class II ERFs to which ZmERF3 belongs have been shown to be repressors of stress-responsive gene expression (Ohta et al., 2001). The parallel induction of a possible activator ZmDREB2A and possible repressor ZmERF3, which both might regulate GCC-box-dependent transcription, seems at first contradictory. But this is probably important for the fine tuning of gene expression of downstream genes as suggested in Arabidopsis where wounding induced the repressing transcription factor AtERF4 as well as the activating transcription factors AtERF1, 2 and 5 (Fujimoto et al., 2000).

The function of ZmCOI6.1 is unknown. Expressed sequence tags (ESTs) from different plant species treated with different stresses such as salt, cold and drought were found to share high sequence similarity to ZmCOI6.1 (i.e. DW042451, CF761062 and CF761080). This indicates that ZmCOI6.1 might be a general stress response gene.

The induction of three of the ZmCOI genes (ZmCOI6.1, ZmACA1, ZmDREB2A) by DMSO treatment at 25 °C supports the observation that changes in the physical properties of the plasma membrane might be the primary signal for cold sensing in maize. These findings are in accordance with the induction of the Medicago sativa cold induced gene cas30 (Örvar et al., 2000) and Brassica napus gene BN115 by DMSO at 25 °C (Sangwan et al., 2001). However, since ZmERF3 was not induced upon DMSO treatment but after exposure of the seedling to low temperature in the dark, membrane fluidity seems not to be the solitary way of the plant to sense a decrease in temperature. All together, a simplified model for a cold stress signaling path can be envisaged placing the regulation of the studied ZmCOI genes, with the exception of ZmERF3, downstream of changes in the membrane fluidity and of the stress signaling molecules JA, ABA, SA and H$_2$O$_2$. Whether these signaling molecules are part of the cold-signaling pathway in maize or whether they act in parallel could not be verified here.

Similar to cold stress, the studied ZmCOI genes were also induced by drought, salinity, heat, high light intensity and wounding indicating some common elements in the signaling pathways for these stresses. Interestingly, the transcript level of most of the studied ZmCOI genes was lower at 24 h than at 6 h of high light intensity and heat stress. Seemingly, the plants were able to acclimate within this period to these stresses and further response was not required, in contrast to the situation during cold, drought and salinity stress.

In this study, we have identified 18 cold induced maize genes. Most of the genes described here have previously not been linked to cold response in maize. The transcription factors ZmDREB2A and ZmERF3 present new possibilities for elucidating the response pathways of this crop to low temperature as well as to other stresses. It still remains to be examined if all the genes induced by cold code for proteins, and what functions, targets and ultimately how the expression and translation are regulated. In particular, the novel gene ZmCOI6.1, which was found frequently replicated in the subtracted cDNA library, needs further characterization.
Chapter 12

ZmCOI6.1, a novel alternatively spliced maize gene whose transcript level changes under abiotic stress*

Maize (Zea mays L.) is an important crop that is poorly adapted to cold stress; very little is known about the components of the response to stress. By means of the cDNA subtraction technique, several novel genes, responsive to cold stress, have been identified, including ZmCOI6.1, the function of which is unknown. The predicted ZmCOI6.1 amino acid sequence and its homologue are very similar to proteins in rice and Arabidopsis, suggesting that it belongs to a conserved group of plant proteins. Analysis of the ZmCOI6.1 promoter sequence revealed several conserved stress-responsive cis-acting elements. Further characterization of expression showed that ZmCOI6.1 was induced not only by cold, but also by drought and salinity as well as by the signaling molecules abscisic acid and salicylic acid, indicating that ZmCOI6.1 is a stress-responsive gene. ZmCOI6.1 is alternatively spliced and yields two transcripts, the level of which change depending on the stress, indicating a possible mechanism of regulation at the splicing level. Constitutive expression of ZmCOI6.1 in Arabidopsis yielded plants that were less tolerant to abiotic stress, providing evidence that ZmCOI6.1 may be a negative regulator.

Introduction

Maize (Zea mays L.) originates from subtropical mid-altitudes of Mexico and is adapted to a semi-arid climate with high light intensity and high temperature during the day and moderate temperature at night (Jaenicke-Despres et al., 2003). The cultivation of maize has been extended to colder regions where damage caused by low temperature is a frequent problem. In particular, temperatures below 15 °C retard germination and seedling growth (Hodges et al., 1997; Hope and Maamari, 1994). Low temperature affects a wide range of processes in the maize seedling; however, the primary cause of this sensitivity to chilling stress is still unknown (for reviews see Leipner and Stamp, 2009; Marocco et al., 2005). Damage caused to maize seedlings by low temperature is primarily to the chloroplasts, leading to inhibition of photosynthesis (Baker et al., 1994). Furthermore, development of maize leaves at low temperature leads to a reduction in the activity of the carbon cycle (Kingston-Smith et al., 1997; Long, 1983) and changes in the antioxidative defenses (Leipner et al., 1997). An important role in this response to low temperature may be played by signaling molecules, such as the plant hormones salicylic acid (SA), abscisic acid (ABA) and jasmonic acid (JA). These plant hormones induce acclimation to cold, resulting in improved tolerance to low temperature (Janda et al., 1999; Janowiak et al., 2002; Lee et al., 1996). Furthermore, hydrogen peroxide (H₂O₂) is regarded as another important signaling molecule and a regulator of expression of some genes in response to stress (Foyer and Noctor, 2005). The response to chilling is accompanied by changes in specific gene transcripts. Some cold-induced genes have been identified in maize, among them genes involved in transcriptional regulation (Qin et al., 2004; Zheng et al., 2006) and in lipid metabolism (Berberich et al., 1998). The wide functional range of maize genes, which respond to cold was also shown by the identification of cold-induced genes by suppression subtractive hybridization (Nguyen et al., 2009). However, the exact molecular function of these genes and encoded proteins in the cold response of maize is still not understood. Most of our knowledge of the molecular response to cold is based on studies of Arabidopsis. In particular, the transcriptional regulation by the C-repeat (CRT)/dehydration-responsive element (DRE) binding factors (CBF) has received considerable attention (Yamaguchi-Shinozaki and Shinozaki, 2006). The identification of the CBF-type transcription factors in other plant species suggests that this transcriptional regulation mechanism is conserved in several plant species, see, e.g., (Choi et al., 2002; Dubouzet et al., 2003). However, there are indications that cold acclimation can also be CBF-independent (Zhu et al., 2004). Beside the regulation on the transcriptional level, gene expression is also regulated by post-transcriptional and post-translational modifications, which seem to be of particular importance during the response to stress.

* based on the following publication:
(Mazzucotelli et al., 2008). Among the post-transcriptional modifications, alternative splicing, by which different forms of mature mRNA are produced from a single transcript, seems to play an important role during abiotic stress (Barbazuk et al., 2008). While the molecular pathways of acclimation of the model plant Arabidopsis to low temperature are well understood, our knowledge of the molecular basis of cold acclimation in maize is still rudimentary. Furthermore, acclimation of Arabidopsis to low temperature stress take place at subzero temperatures, while maize cold-acclimates at temperatures below 20 °C, suggesting that the acclimation pathways may differ. In order to better characterize the molecular pathways that are induced in maize in response to cold stress, cold-induced genes were searched by suppression subtractive hybridization in an earlier study (Nguyen et al., 2009). Among the genes found in this study, the novel gene ZmCOI6.1 was found to be induced by low temperature and other abiotic stress factors as well as by stress signaling molecules. The aim of this study was a detailed characterization of factors as well as by stress signaling molecules. The study reveals that ZmCOI6.1, which is very similar to se-
quences in other plant species, contains several stress responsive cis-acting elements in its promoter. Furthermore, it was found that the expression of ZmCOI6.1 in response to abiotic stress is modified by alternative splicing. Functional analyses of ZmCOI6.1 transformants indicate that it may play a role as a negative regulator.

### Material and methods

#### Plant material and growth conditions
Maize (Zea mays L.) seeds of the genotype ETH-DH7 (Fracheboud et al., 2004) were grown in half-strength Hoagland solution (H2395, Sigma) supplemented with 0.5% Fe-sequestrene, 6 mM K+ and 4 mM Ca2+. Before treatment, plants developed in growth chambers (Conviron PGW36, Winnipeg, Canada) at 25 °C/22 °C (day/night) until the third leaf was fully developed; the photoperiod was 12 h, the light intensity 300 µmol m⁻² s⁻¹ and the relative humidity 60%-70% (day/night).

#### Reverse transcriptase (RT)-PCR, cloning and analysis of cDNA
Total RNA was extracted from maize leaf samples using Tri Reagent® according to Sigma’s protocol for RNA isolation. From each sample, 1.5 µg total RNA was reverse-transcribed to first-strand cDNAs using oligo (dT)23 primer (Table 1) in a total volume of 20 µl, according to the supplier’s instructions (Advantage RT-for-PCR Kits, DB Biosciences, Clontech). Synthesized cDNAs were diluted in 100 µl H2O; 4 µl diluted cDNA in a volume of 20 µl were used as templates for PCR amplification: 25 circles at 95 °C for 30 s, at 57 °C for 30 s and at 72 °C for 60 s and finally at 72 °C for 5 min. The maize ubiquitin transcript, ZmUBI (accession number S94466), was the internal standard. Amplified PCR products (15 µl) were separated by electrophoresis on 2.0% (w/v) agarose gel, monitored by means of Gel Doc 2000 (Bio-Rad Laboratories, Hercules, CA, USA) and quantified by the software ImageJ 1.41 (National Institute of Mental Health, Bethesda, Maryland, USA). The cDNA from the PCR amplification was cloned into the pDrive vector (QIAGEN GmbH, Hilden, Germany) and transformed into E. coli DH5α cells. Clones were sequenced by MWG (MWG-Biotech AG, Ebersberg, Germany).

#### Abiotic stress and signaling molecules
Abiotic stress or signaling molecules were applied to maize plants when their third leaf was fully developed. The plants were cold-stressed by decreasing the temperature to 6 °C or 13 °C. For the drought stress, maize plants were removed from the hydroponic culture and were left to dry in a growth chamber. The salt treatment was induced by adding sodium chloride to the Hoagland solution to obtain a final concentration of 150 mM. Stress signaling molecules were added to the hydroponic culture to achieve a final concentration of 100 µM salicylic acid (SA) or 100 µM abscisic acid (ABA); the concentrations were chosen based on their efficacy found in own and others studies (Hu et al., 2005; Janda et al., 1999; Nguyen et al., 2009; Senaratna et al., 2000). All the treatments were conducted in the dark. Control plants (unstressed) were collected before applying the stress treatments. The middle part of third leaf was harvested, frozen in liquid nitrogen and stored at -80 °C until assay.

### Table 1: Oligonucleotides used to sequence ZmCOI6.1 and for RT-PCR analysis.

<table>
<thead>
<tr>
<th>ID</th>
<th>Primer sequence</th>
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<tr>
<td>9909-2</td>
<td>5'-CGCTGTTGCAGCTAGAAATAG-3'</td>
</tr>
<tr>
<td>6551-2</td>
<td>5'-TAGCCCCGCTGACGCGTGC-3'</td>
</tr>
<tr>
<td>ZmCOI6.1_F</td>
<td>5'-CGTACGGTACTCTCTGAG-3'</td>
</tr>
<tr>
<td>ZmCOI6.1_R</td>
<td>5'-CGTGGAGGATCCATCGGAC-3'</td>
</tr>
<tr>
<td>ZmCOI6.1b_F</td>
<td>5'-GTTTGATACAGATCCGCC-3'</td>
</tr>
<tr>
<td>ZmCOI6.1b_R</td>
<td>5'-AGTGCCCCAGAAATGTGGAC-3'</td>
</tr>
<tr>
<td>C6.1-4</td>
<td>5'-CATGAGTCCCTATCATCACGCC-3'</td>
</tr>
<tr>
<td>C6.1-5</td>
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<td>AtCOI6.1-3</td>
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<tr>
<td>AtCOI6.1-2</td>
<td>5'-GTGTTGTAATCGTGTTAGA-3'</td>
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</tbody>
</table>
Among 44 BASTA-resistant T1 lines, three were above. Drought and salt was estimated by measuring the seven days after the first salt treatment. Tolerance to stress was applied by immersing four-week-old plants (including their containers) into 150 mM NaCl for 30 min. Dehydration was induced by leaving four-week-old plants fully immersed in water and a water film covered the leaf. The samples were incubated in a refrigerated circulator (FP40-HP, Julabo, Germany) at 0 °C for 1 h. Then, the temperature was decreased by 2 °C per hour to -10 °C. At certain temperatures, the electrolyte leakage was measured after boiling 200 µl deionized water, so that the petioles were fully immersed in water and a water film covered the leaf. The samples were incubated in a refrigerated circulator before and subsequent incubation for 30 min. with a conductometer (Metrohm, Herisau, Switzerland). Maximum electrolyte leakage was determined after thawing and subsequent incubation for 30 min. with a conductometer (Metrohm, Herisau, Switzerland). Maximal electrolyte leakage was measured after boiling for 30 min. Dehydration was induced by leaving four-week-old plants without water for seven days. Salt stress was applied by immersing four-week-old plants (including their containers) into 150 mM NaCl for 5 min. This treatment was carried out twice a day on two consecutive days; plant traits were measured seven days after the first salt treatment. Tolerance to drought and salt was estimated by measuring the electrolyte leakage of the whole plants as described above.

Bioinformatics
A similarity search was performed with the basic local alignment search tool (BLAST) at the National Centre for Biotechnology Information (NCBI, Bethesda, MD, USA) (http://www.ncbi.nlm.nih.gov/BLAST/) and the NCBI BLAST2 service maintained by the Swiss Institute of Bioinformatics (http://au.expasy.org/tools/blast/). PLACE (http://www.dna.affrc.go.jp/PLACE/), a database of motifs found in plant cis-acting regulatory DNA elements, was used to scan the promoter of the ZmCOI6.1 gene. Prediction of splicing was realized by means of the Genescan program (http://genes.mit.edu/GENSCAN.html). The phylogenetic tree was constructed by the program CLUSTAL W. Analysis of the amino acid sequence for transmembrane regions was carried out using TMPred (Hofmann and Stoffel, 1993).

Results
ZmCOI6.1, a novel cold-induced gene is conserved in plant species
In a previous study of the chilling-tolerant maize genotype ETH-DH7, several novel cold-induced genes were identified (Nguyen et al., 2009). One of these genes, namely ZmCOI6.1, which was represented by four different cloned fragments, was further characterized because of its frequent occurrence in the screening. To determine the complete sequence of ZmCOI6.1, oligonucleotides were designed, which covered the AZM4_69676 sequence from the maize genotype B73 that showed 96% homology with the ZmCOI6.1 fragments. The oligonucleotides were chosen so that only this sequence, not the AZM4_12960 homologue sequence, was amplified; the latter showed 81% homology with the ZmCOI6.1 fragments (for primer sequences see Table 1). Overlapping regions of the corresponding gene in the ETH-DH7 genotype were amplified, and the overlapping fragments were sequenced, assembled and annotated in the NCBI GenBank (accession number DQ060243) (Figure 1).

Databases were searched to determine the possible existence of homologues and/or orthologues of the predicted amino acid sequence of ZmCOI6.1. Nine amino acid sequences, similar to the ZmCOI6.1 sequence, were identified: one maize homologue, two rice (Oryza sativa L.) orthologues (Os03g13810 and Os10g03550 in the TIGR rice genome annotation database, http://www.tigr.org) and six Arabidopsis thaliana orthologues (At1g20100, At1g75860, At2g17787, At3g07280, At4g35940 and At5g48610). Furthermore, ZmCOI6.1 had a similar nucleotide sequence as expressed sequence tags (EST) from wheat (Triticum aestivum L.), barley (Hordeum vulgare L.), sugarcane (Saccharum officinarum L.) and sorghum (Sorghum bicolor L.) (data not shown).

Based on the predicted amino acid sequences, the phylogenetic relationship among sequences derived from maize, rice and Arabidopsis were analyzed (Figure 2). This analysis revealed three main groups: (i) ZmCOI6.1, the maize homologue sequence AZM4_12960 and the rice sequences, (ii) the Arabidopsis sequences At4g35940, At2g17787, At3g07280 and At5g48610 and (iii) At1g75860 and At1g20100.
The amino acid sequence for transmembrane regions before, suggesting that they are novel. Analysis of The function of these motifs has not been described was identified at the N-terminal of all the sequences. [FY]-[TV]-V-P-F. Furthermore, a lysine-rich region gion was the C-terminal with the putative motif L-P- at the N- and C-terminals. The most conserved re-

quences were compared; similar domains were found in rice (Os) and Arabidopsis (At) was constructed using the program CLUSTAL W.

by TMpred did not reveal the presence of transmembrane domains, suggesting that ZmCOI6.1 is a soluble protein.

ZmCOI6.1 gene is alternatively spliced

Two fragments were obtained upon RT-PCR analysis of the ZmCOI6.1 transcript and not the expression products of another gene or other genes, both forms were cloned using the oligonucleotides 6551-2 and ZmCOI6.1b_R and subsequently sequenced. The sequence analysis revealed that both cDNA forms, named ZmCOI6.1-a and ZmCOI6.1-b, were identical with the specific parts of the ZmCOI6.1 gene. The analysis of the putative spliced forms of ZmCOI6.1 indicated that ZmCOI6.1-b expanded from nucleotide 640 to nucleotide 3196 with 101 nucleotides missing between positions 2179 and 2281, i.e. intron 2 (I2). In the ZmCOI6.1-a transcript, the regions between 744 and 1867, i.e. intron 1 (I1), and between 2179 and 2281 were missing (Figure 3).

To identify the positions of the introns and exons as well as the splicing points, the ZmCOI6.1 sequence was analyzed for the splicing consensus sequence of the introns 5’-splice donor site (AG/GTAAGT) and of the 3’-splice acceptor site (TGCGAG/G) as well as the consensus branch point region (CURAY) (R, purine; Y, pyrimidine) (Lorković et al., 2000). Both the first and second introns had a conserved 5’-splice donor region for the putative spliced forms of the ZmCOI6.1 gene. The putative spliced forms of ZmCOI6.1 indicated that ZmCOI6.1-b expanded from nucleotide 640 to nucleotide 3196 with 101 nucleotides missing between positions 2179 and 2281, i.e. intron 2 (I2). In the ZmCOI6.1-a transcript, the regions between 744 and 1867, i.e. intron 1 (I1), and between 2179 and 2281 were missing (Figure 3).

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Figure 2: ZmCOI6.1 putative amino acid sequence shares similarities with sequences in Oryza sativa and Arabidopsis thaliana. The phylogenetic tree of the amino acid sequences of the deduced ZmCOI6.1 amino acid sequence and its homologue (AZM4_12960) and orthologue sequences in rice (Os) and Arabidopsis (At) was constructed using the program CLUSTAL W.

Figure 1: The nucleotide sequence of the genomic region covering the ZmCOI6.1 gene including the predicted promoter region with its putative stress responsive cis-acting elements (for details see Table 2). Capital letters indicate exons of the putative ZmCOI6.1 gene product. Introns and non-coding regions are represented by lower-case letters. The start (ATG) and stop (TAA) codons are bold and italics. Spliced donor modules in intron I1 and I2 are underlined (=). The spliced acceptor site in intron 12 is underlined (+).
suboptimal temperature (13 °C) on the expression of clearly present again at 24 h. To test the effect of the experimental set-up was the same as for the was retained in one of 0.5 kb (At4g35940-ß).

Alternative splicing of the At4g35940 gene under different abiotic stresses and with different signaling molecules

To better understand the cold induction of the ZmCOI6.1 gene, an experiment was conducted, in which seedlings were exposed to 6 °C for 24 h and samples were collected after 1, 2, 4, 6, 12 and 24 h to analyze early and late responses. The semi-quantitative RT-PCR revealed that ZmCOI6.1-α and ZmCOI6.1-ß were expressed at time 0 (control) and under cold stress (Figure 4). A lower level of ZmCOI6.1-ß was found under the control conditions, but the levels increased with time under cold stress. The smaller cDNA form, ZmCOI6.1-α, decreased during the first 12 hours of exposure to 6 °C but was clearly present again at 24 h. To test the effect of suboptimal temperature (13 °C) on the expression of ZmCOI6.1 and the expression of the two fragments, the experimental set-up was the same as for the treatment at 6 °C. At 13 °C, the ZmCOI6.1-α transcript was also present and remained stable over time, while the levels of ZmCOI6.1-ß increased and stayed afterwards at a constant level (Figure 4). These results show that ZmCOI6.1 is induced after short exposure to cold and increases with time.

In a previous study (Nguyen et al., 2009), the expression of ZmCOI6.1 was studied after exposure of Arabidopsis plants to 4 °C. The RT-PCR was conducted with the primers ZmCOI6.1-α and ZmCOI6.1-ß. The ubiquitin gene was tested upon exposure to 4 °C and ß. The ubiquitin gene was tested upon exposure to 4 °C (A) and 13 °C (B) and induction of its Arabidopsis orthologue At4g35940 upon exposure to 4 °C (C). 0 h indicates samples taken prior to treatment. RT-PCR was performed with the primers 6551-2 and ZmCOI6.1b_R to analyze the expression of the ZmCOI6.1 transcript forms ZmCOI6.1-α and ZmCOI6.1-ß and with the primers AtCOI6.1-3 and AtCOI6.1-2 to analyze the expression of the At4g35940 transcript forms α and β. The ubiquitin genes ZmUBI and AtUBI were used as internal control. Bands were quantified in relation to the expression of ZmCOI6.1-α at time 0 h.
transcript was suppressed.

The ZmCOI6.1 gene promoter contains predicted conserved stress cis-acting elements

Genes that are induced by stress or other treatments usually harbor short sequences, cis-acting elements, within the promoter, which are identified by transcription factors, thus regulating gene expression. To analyze the ZmCOI6.1 promoter, cis-acting elements were searched in databases with the program PLACE. Several cis-acting elements were identified in the ZmCOI6.1 promoter, including the low temperature-responsive elements MYC, DRE/CRT-core, DRE/CRT-HvCBF2, LTRE-core and LTRE-1. Other cis-acting elements involved in abiotic and biotic stress were identified: MYB1, ABRE-like G-box, MYB-core and ASF1 (Figure 1; Table 2). Some of these cis-acting elements were also present in the promoter of the orthologues of ZmCOI6.1, suggesting that they share a common feature of possible transcriptional regulation (data not shown).

Arabidopsis 35S::ZmCOI6.1-a transgenic plants were more sensitive to abiotic stress than wild-type plants

In order to obtain information about the function of ZmCOI6.1, transgenic Arabidopsis plants, which constitutively expressed the fully spliced form (ZmCOI6.1-a) of ZmCOI6.1, were produced. The tolerance of the 35S::ZmCOI6.1-a transgenic and wild-type plants to freezing, with and without previous acclimation to cold, was determined on leaves of four-week-old plants. All the transgenic lines dis-

sponses. The ZmCOI6.1 gene transcript accumulated under drought and under NaCl stress as well as after treatment with SA and ABA (Figure 5). The strongest induction was obtained during drought and during the ABA treatment, where the ZmCOI6.1-ß transcript forms increased but ZmCOI6.1-α did not. In the NaCl and SA treatments the expression of the ZmCOI6.1-α transcript was suppressed.

Table 2: Putative stress-responsive cis-acting elements in the ZmCOI6.1 promoter region. The table lists the identified cis-acting elements (as presented in Figure 1) including recognition motifs and induction specificity. The promoter was analyzed with PLACE (http://www.dna.afrc.go.jp/PLACE/) to identify putative transcriptional regulatory motifs. 1-as-1-like elements are characterized by two imperfect TGACGCTGA motifs, separated by 4 bp. 2-11-bp ethylene-responsive element, TAAGAGCCGCC, 3-G-box is 5'-C/A-ACACGTGGCA-3' with a CACGTG hexanucleotide core. 4K = G or T; N = A, C, G or T; R = A or G; W = A or T; Y = C or T.

cis-Acting element | Recognition sequence | Involvement (reference)
--- | --- | ---
MYC | CANNTG | Drought (ABA) (Abe et al., 1997), cold (Pasquali et al., 1999)
MYB-core | CNGTTR | Drought stress (Urao et al., 1993)
MYB1 | WAACCA | Dehydration stress (ABA) (Abe et al., 2003)
MYB2 | YACGG | Dehydration stress (ABA) (Abe et al., 2003)
ABRE-like | ACCTG | Drought (etiolation) (Simonson et al., 2003)
DRE/CRT-core | TACCCGAC | Cold- and dehydration-responsive expression (Dubouzet et al., 2003)
DRE/CRT-HvCBF2 | GTCGAC | Low temperature (Xue, 2003)
DRE1-Rab17 | ACCCGAG | Drought (ABA) (Busk et al., 1997)
LTR-core | CCGAC | Low temperature (Jiang et al., 1996)
LTR-1 | CCGAAA | Low temperature (Dunn et al., 1998)
ASF1 | TGACG | Auxin and/or salicylic acid; perhaps light regulation (Terzaghi and Cashmore, 1995)
INR | YTANTCGY | Light-responsive (Nakamura et al., 2002)
AGC-box or GCC-box | AGCCGCC | Ethylene (= ethylene-inducible defense genes) (Ohme-Takagi et al., 2000)
GCC-core | GCCGCC | Defense (Brown et al., 2003), jasmonate (Chakravarty et al., 2003)
ACGT | ACCTG | Drought (etiolation) (Simonson et al., 2003)
G-box | CAGGTG | Pathogen (Pasquali et al., 1999), ABA (Guiltinan et al., 1990), light (Giuliano et al., 1988)
Realpha | AACCAAA | Etiolation (Degenhardt and Tobin, 1996)
HSE-like | CNNGAANNNTCTNN | Heat shock (Schoffl et al., 1989), pathogen (Pflitzner et al., 1988)
played a decreased tolerance to freezing compared to wild-type plants despite their level of cold acclimation (Figure 6). The sensitivity of the three transgenic lines to drought as well as to NaCl was estimated from electrolyte leakage of the leaf tissue after stress. The transgenic and wild-type plants, grown under optimal conditions, did not show significant differences in initial electrolyte leakage (data not shown). After seven days of water shortage, the wild-type plant showed no signs of wilting compared to the 3SS::ZmCOI6.1-α transgenic plants, which showed clear symptoms of severe drought stress and significantly higher electrolyte leakage than the wild-type plants (Figure 6). A significant difference was also observed between transgenic lines and wild-type plants after treatment with NaCl. After seven days of the NaCl treatment, the electrolyte leakage from the leaves of 3SS::ZmCOI6.1-α transgenic lines increased to about 60%, whereas the leakage from wild-type plants was 42% (Figure 6).

**Discussion**

In order to gain more insight into the response of maize to low temperature, ZmCOI6.1, a novel cold-induced gene was characterized. The sequence analysis revealed that ZmCOI6.1 is a conserved gene in plants showing very high similarity to sequences from rice and Arabidopsis and to ESTs from wheat (Triticum aestivum L.), barley (Hordeum vulgare L.), sugarcane (Saccharum officinarum L.) and sorghum (Sorghum bicolor L.). From the deduced amino acid sequences, it is assumed that these proteins are soluble. Furthermore, they share several motifs of high identity, the function of which has yet to be characterized.

The ZmCOI6.1 gene was induced by several abiotic stresses and by signaling molecules, indicating that ZmCOI6.1 is probably involved in the general stress response (Nguyen et al., 2009). This was also supported by the fact that its promoter contains several cis-acting elements, suggesting possible regulation
The retention of unspliced introns in a fraction of the transcripts seems to be common in plants and may either reflect inefficient splicing or a regulatory process (Lorković et al., 2000). In support of the latter, it was found in Arabidopsis that retained introns were a large portion of the alternatively spliced forms (Ner-Gaon et al., 2004). The transcripts with retained introns were, for the most part, transcripts of stress and external/internal stimuli-related genes. For example, an intron retention mechanism has been described for cold-regulated genes in durum wheat (Mastrangelo et al., 2005); genes coding for a putative ribokinase and a C3H2C3 RING-finger protein were characterized by the stress-induced retention of a subset of introns in the mature mRNA.

The balance between ZmCOI6.1-α and ZmCOI6.1-β was affected by the extent of the temperature stress. While at 13 °C similar levels of both forms were transcribed, at 6 °C there was more ZmCOI6.1-β than ZmCOI6.1-α. This difference in the transcript levels at 6 °C and 13 °C suggests that alternative splicing may play an important role in the regulation of ZmCOI6.1 expression, depending on the extent of the low-temperature stress. Under a strong cold stress (6 °C), ZmCOI6.1-α was down-regulated or remained constant. Therefore, to compensate for the induction of the gene through the cis-acting elements in the promoter, alternative splicing is favored to produce ZmCOI6.1-β, a transcript that leads to the truncated polypeptide. The presence of ZmCOI6.1-α after 24 h exposure to 6 °C seems to be caused by the high transcription rate of ZmCOI6.1 and, consequently, a high amount of ZmCOI6.1 pre-mRNA that may result in the splicing of some of the ZmCOI6.1 pre-mRNA. On the other hand, under moderate stress (13 °C), the function of ZmCOI6.1 may be more important; alternative splicing would shift towards the fully spliced transcript ZmCOI6.1-α, as shown by its increase at 13 °C, in contrast to 6 °C.

Consequently, the production of the two splicing forms may be important for regulating the amount of the ZmCOI6.1 protein. Such regulation has been proposed for the flowering gene FCA in Arabidopsis (Quesada et al., 2003) and seems to be common in eucaryotes (Maquat, 2004). The type of ZmCOI6.1 transcript regulation and the presence of stress responsive cis-elements suggest that ZmCOI6.1 may act as a stress-responsive regulator. The analysis of Arabidopsis plants constitutively expressing ZmCOI6.1-α gave first evidence of the potential function of ZmCOI6.1. The greater susceptibility to stress of these plants compared to wild-type plants indicates that ZmCOI6.1 may function like a negative regulator. This assumption is supported by the observation that T-DNA insertion in At4g35940, the Arabidopsis orthologue of ZmCOI6.1, resulted in plants that are more tolerant to cold, drought and salt stress compared to wild-type plants.
(data not shown). With regard to ZmCOI6.1, however, we still do not know how alternative splicing regulates the activity of ZmCOI6.1 and the precise role of ZmCOI6.1 in the response of maize to low temperature stress.
Chapter 13

General discussion and outlook

General discussion

Development of the photosynthetic apparatus at suboptimal temperature

Seedling development at suboptimal temperature seems to be the crucial point in respect to the chilling tolerance of maize, whilst sudden cold stress rarely affects field grown maize in the temperate regions of Europe. Moreover, the physiological experiments have shown that the symptoms of sudden cold stress seem to be largely reversible in fully developed leaf tissue (Leipner et al., 2000a). In particular, the development of a functional photosynthetic apparatus appears to be the bottleneck for seedling growth under chilly conditions. The comparison of genotypes of contrasting chilling tolerance (Chapter 3) and the QTL analyses (Chapter 4 and following) support this view. The major corroborative arguments are: (i) differentiation of maize seedlings with contrasting chilling tolerance is only feasible when seedlings develop under a mild chilling stress (suboptimal temperature), whilst sudden cold stress in plants grown at optimal temperature equally affects the different genotypes despite their differences in chilling tolerance, and (ii) the expression of the major QTLs for chilling tolerance of photosynthesis is consistent between controlled growth at suboptimal temperature and early development in the field.

Theoretically, photosynthetic activity can be increased by increasing the amount of absorbed light energy, i.e. by raising the chlorophyll content, and/or by increasing the quantum efficiency of the photosynthetic apparatus. Investigating the interaction between traits using QTL analysis revealed that the functioning of the photosynthetic machinery and the content of chlorophyll can be independently regulated. For the plant breeder, this implies that the selection of seedlings with greener leaves does not necessarily guarantee obtaining plants with a better photosynthetic performance. Changes in the chlorophyll $a/b$ ratio seem to be a characteristic response to alterations in growth temperature. It was found that the chlorophyll $a/b$ ratio decreases when maize develops at suboptimal temperature (Haldimann et al., 1995), especially in chilling-sensitive genotypes (Haldimann, 1998). Furthermore, QTLs for chilling tolerance of photosynthesis were associated with QTLs for the chlorophyll $a/b$ ratio (Chapter 9; Fracheboud et al., 2002). A shift in the chlorophyll $a/b$ ratio indicates an alteration of the ratio between PSII reaction center (RC) and light harvesting complex (LHC). The lowered chlorophyll $a/b$ ratio indicates that maize seedlings grown at suboptimal temperature are characterized by a lower ratio of RC to LHC in comparison to seedlings grown at optimal temperature. It can be assumed that such a decrease in the RC:LHC ratio increases the excitation pressure on each RC; a circumstance that should be avoided especially under stress conditions.

This type of response seems to be specific for maize when it is exposed to low temperature. Under drought stress, the chlorophyll $a/b$ ratio of maize seedlings increases especially in the mesophyll cells (Alberte and Thornber, 1977). The study of Alberte and Thornber also indicated that the size of the photosynthetic unit decreases when plants were exposed to water deprivation. Also under short-term salt stress, the chlorophyll $a/b$ ratio of maize seedlings tends to increase with increasing salt concentration (Shabala et al., 1998). In contrast, growth at low temperature does not affect the chlorophyll $a/b$ ratio of winter hardy species, such as pine and winter wheat (Savitch et al., 2002).

The different response of the chlorophyll $a/b$ ratio to cold as opposed to drought and salinity stress indicates that the decreased ratio of RC to LHC is a problem rather than a strategy during growth at low temperature. Moreover, it implies that low temperature stress mainly acts on the development of the photosynthetic apparatus in maize. This assumption is supported by studies on intermittent light (IML) grown maize seedlings (Box 1). Growth at IML induces the formation of reaction centers (RC), but hinders the accumulation of light harvesting complexes (LHC). Growth at low temperature under IML, however, results in plants that are photosynthetically inactive, indicating that RCs can not be accumulated or are not functioning under such a condition. In contrast, the accumulation of LHCs takes place also at low temperature.
The low number of (functional) PSII reaction centers in seedlings that developed at suboptimal temperature can have at least two reasons: (i) reaction centers are not synthesized or not integrated into the thylakoid membrane or (ii) the turnover of the D1 protein is disturbed. The D1 protein, which is an important compound of the PSII reaction center, is encoded by the chloroplast, which would exclude genotypic differences such as found here. However, de novo synthesis, targeting and integration into the thylakoid membrane as well as the turnover of the D1 protein involves several steps that are regulated or catalyzed by nuclear encoded proteins (Mulo et al., 2008). A precise analysis of the kinetics of recovery from photoinhibition under low temperature would be necessary to elucidate the affected step. It is rather uncertain that a disturbed D1 protein turnover could affect the chlorophyll a/b ratio.

Genotypic differences in the chlorophyll a/b ratio between chilling-tolerant and chilling-sensitive genotypes grown at suboptimal temperature are present only at moderate or high light intensity but not at low light intensity during growth. However, both groups considerably differed in their photosynthetic activity also at low light intensity (Haldimann, 1998). This implies that the chilling tolerance of photosynthesis seems to be not solely associated with an altered ratio of RC to LHC. Earlier physiological studies pointed to the direction that the activity of certain enzymes of the C4 cycle (Long, 1983) or the capacity of the antioxidative defense mechanisms (Foyer et al., 2002) might be the key for the chilling tolerance of the maize seedling. Furthermore, anthocyanins have been found to provide protection against chilling induced photoinhibition (Pietrini et al., 2002). In order to elucidate whether these compounds are responsible for genotypic differences in chilling tolerance of photosynthesis, co-localizations of QTLs for antioxidants and C4 cycle enzymes with QTLs found for chilling tolerance of photosynthesis were
searched for. Neither the QTLs for the activity of C₄ cycle enzymes and for antioxidants (Chapter 9) nor the QTLs for anthocyanin content (Chapter 8) were found in the same genomic region as QTLs for chilling tolerance of photosynthesis. Furthermore, QTLs for acid vacuolar invertase were not associated with QTLs for chilling tolerance of photosynthesis (Chapter 8). Based on these results it can be concluded that the above mentioned components are not responsible for the genotypic differences in chilling tolerance of photosynthesis, at least not in the studied material and under the chosen conditions. However, it can not be ruled out that in other populations pleiotropic effects between these traits may exist. Furthermore, it must be considered that the greening tissue is the most vulnerable part (see Chapter 1) and that we currently do not know whether genotypic differences for antioxidants, C₄ cycle enzymes or anthocyanin may be present in this tissue. However, the results obtained here clearly demonstrate that a contrasting chilling tolerance of photosynthesis may exist without an involvement of C₄ cycle enzymes, antioxidants or anthocyanin.

The influence of light intensity on the alteration of the chlorophyll a/b ratio due to suboptimal growth temperature underlines that it is highly important to consider irradiance when studying chilling stress. In respect to the chlorophyll content, a higher light intensity aggravates the effects of suboptimal growth temperature in chilling-sensitive genotypes (Haldimann, 1998); however, the higher amount of xanthophyll cycle pigments and their higher de-epoxidation state under this condition suggest that growth at high light intensity may enhance the tolerance against further photooxidative stress. Whatever the primary molecular cause of the genotypic differences in the chilling tolerance of photosynthesis, high light intensity itself can also induce the expression of major QTLs for chilling tolerance of photosynthesis (Chapter 7). Moreover, the expression of the major QTL for chilling tolerance of photosynthesis at chromosome 6 of the ETH-mapping population was even found under favorable field conditions though with only a very small additive effect (Chapter 5). It can be assumed that this QTL is in fact constitutively expressed, but a strong QTL effect is only visible when plants are exposed to a higher excitation pressure, be it due to chilling stress in the light or be it due to high light intensity. It remains open whether this QTL is also expressed by low temperature per se, i.e. under low temperature in combination with low light intensity. In order to test this, an analysis would require the consideration of possible secondary effects caused by a shortage of assimilates which is unavoidable under such a condition. The results obtained in the studies of QTL expression under different cold temperatures at night, point to the direction that temperature per se seems to affect the expression of QTLs (Chapter 8). The identification of several QTLs for chilling tolerance of photosynthesis strongly suggests that there is not a single bottleneck, which makes a chilling-sensitive genotype susceptible to low temperature. In fact there are several factors that may be responsible for chilling sensitivity.

**Potential QTL candidate genes**

The sequencing of the maize genome is very advanced; both the physical as well as the genetic map position of many genes are now known. Consequently, the identification of positional candidate genes, which can additionally also explain the phenotype by the function of their gene product, is feasible. However, the verification of such a candidate gene by conducting a QTL analysis for its gene product often seems to give negative results, as seen in Chapter 8. The large sum of genes in a certain QTL region makes it very difficult to find a particular gene responsible for the expression of QTLs for complex traits. Even when the number of positional candidate genes was restricted by extensive phenotypic analyses of the studied QTL, it is still similar to searching for a needle in a haystack. Nevertheless, QTL analyses for the activity of certain enzymes often discover QTLs which closely map to their gene (e.g. Chapter 8; Prioul et al., 1999). In such a case, a very high probability exists that variations in these genes or their promoter sequences are responsible for the expression of the QTL. The identification of QTLs for biochemical traits, e.g. enzyme activity, bears the great potential to study the effects of this enzyme on other traits by looking for pleiotropic effects at the particular genomic region. Such a strategy is interesting when mutants are not available or effects in the range of natural variation are intended to be studied.

Bearing in mind the shortcomings for the identification of candidate genes, current versions of consensus maps, in particular of the IBM Neighbors map, were scanned for potential candidate genes which could explain the phenotype of the major QTLs for chilling tolerance of photosynthesis. Most of these candidate genes are involved directly or indirectly in photosynthesis. In-depth analysis of the traits, which are affected by these QTLs, restricted the number of candidate genes to genes that are supposedly involved in the assembly or regulation of the photosynthetic apparatus, i.e. of the light reaction of photosynthesis.

Exemplified by the main QTLs for chilling tolerance of photosynthesis on chromosomes 2 and 6, some candidates have been identified. Based on the IBM2 Neighbors consensus genetic map, the gene hcf106 is present at the chilling tolerance locus on chromosome 2. This gene codes for the high chlorophyll fluorescence protein 106, which is a component of
the ΔpH-dependent translocation pathway in the thylakoid membrane (for review see Mori and Cline, 2001). Nuclear mutation of hcf106 results in a pale green, non-photosynthetic seedling, which emits absorbed light energy as chlorophyll fluorescence (Martienssen et al., 1989). This result is similar to maize seedlings grown at suboptimal temperature and carrying the allele at this locus from the chilling-sensitive line.

Candidate genes, which were identified to be located in the vicinity of the QTL on chromosome 6 of the ETH mapping population, were part of the photosynthetic machinery or involved in the turnover of the D1 protein. One of these genes is psaH, which codes for PSI-H, a subunit of PSI. The PSI-H seems to play an important role in the state transition as was found in mutants of Arabidopsis with low levels of psaH (Lunde et al., 2000). Another is cab-m7, coding for the LHCII protein Lhcbm7 which is preferentially expressed in maize mesophyll cells and is strongly induced upon illumination (Becker et al., 1992).

Among published ESTs within this QTL region, another candidate gene was identified. This EST (PCO153568) shows homology with the serine protease DegP1 from Arabidopsis. It is well known that DegP1 plays an important role in the repair of degraded D1 protein of the PSII reaction center (Kapri-Pardes et al., 2007). Since Fv/Fm is strongly affected by integrity of the D1 protein (Salonen et al., 1998), alterations in the amount or the activity of the DegP1 could explain the changes in Fv/Fm. However, it remains to be elucidated whether one of these genes is responsible for the expression of the main QTLs for chilling tolerance of photosynthesis.

Molecular studies have the potential to support the search for candidate genes, because genes that are strongly up-regulated in plants exposed to stress conditions are promising candidates. The map positions of the cold-induced genes, which were identified in the study presented in Chapter 11, were searched in silico and compared to the positions of the major QTLs for chilling tolerance of photosynthesis. This analysis did not result in co-localization (data not shown). However, it must be mentioned that it would be necessary to map these genes on the real genetic map.

Another possibility to identify potential candidate genes is to search for genes that are differentially expressed in the parental lines when exposed to low temperature. Microarray analysis using mRNA of the parental lines of the ETH population revealed several genes that were induced more in ETH-DH7 than in ETH-DL3 when seedlings were subjected to cold stress. In particular, these were genes involved in lipid and membrane metabolism, antioxidative defense and regulation of transcription and also genes whose gene products are directly involved in photosynthesis (personal communication P. Sowinski, University of Warsaw). So far, data analyses have revealed a late embryogenesis abundant protein gene (mlg3) which is stronger induced under cold conditions in ETH-DH7 than ETH-DL3 and which is located within the confidence interval of the QTL for chilling tolerance of photosynthesis on chromosome 6.

Interaction between chilling stress and other abiotic stresses

Cross interactions between different abiotic stresses are likely and were documented, e.g., in maize for the interaction between drought and chilling stress (Irigoyen et al., 1996). The early molecular response to cold stress in maize seedlings seems to involve the same genes, which are also expressed in response to other abiotic stresses (Chapter 11). This molecular network is well accepted in Arabidopsis (Yamaguchi-Shinozaki and Shinozaki, 2006) and could be verified in parts here also in maize seedlings. However, there seem to be differences between species in respect to the precise function or position within the network of some of the genes as seen for the DREB2 orthologue of maize (Chapter 11). A part of this network seems to be the gene ZmCOI6.1 that appears to function as a negative regulator (Chapter 12).

Our own experiments have shown that the chilling-tolerant line ETH-DH7 is characterized by higher drought and salt tolerance than ETH-DL3 (data not shown). However, it is still uncertain whether a co-selection for these traits occurred or whether the same underlying mechanism exits. The QTL analyses of the ETH-population revealed that QTLs for chilling tolerance of photosynthesis and QTLs for drought tolerance, as assessed by leaf rolling under water limitation, were not located within the same chromosomal regions (see also Figure 2). However, it must be mentioned here that chilling tolerance and drought tolerance were determined at different growth stages.

A pleiotropic effect between chilling tolerance and drought tolerance was implicated by the comparison of QTL analyses of another mapping population. The Ac7643 × Ac7729 population, which was developed to study drought tolerance, also segregates for chilling tolerance and the major QTL for chilling tolerance of photosynthesis (Fracheboud et al., 2002) mapped at the same position as a QTL for drought tolerance of photosynthesis (personal communication R. Messmer, ETH Zurich) and as a QTL for plant height under low nitrogen content in both well watered and drought stress conditions (Ribaut et al., 2007).

In a third example, the IBM302 population, which was here analyzed for QTLs associated with chilling tolerance of photosynthesis (Chapter 8), was examined by us for drought tolerance under controlled conditions as well as in the field. The comparative analysis of the results, however, did not reveal com-
mon QTLs (data not shown). Seemingly, genetic variations in drought tolerance and genetic variations in chilling tolerance have a different origin. This means that although drought stress seems to induce chilling tolerance it does not necessarily mean that a drought tolerant genotype is more chilling-tolerant.

**Genetic variations for chilling tolerance**

From earlier studies, it was suggested that there is a considerable genetic variation for chilling tolerance within the maize germplasm (Bhosale et al., 2007; Greaves, 1996; Lee et al., 2002a). The comparison of different mapping populations, in respect to QTLs for chilling tolerance, revealed that most of the chromosomes are involved in the expression of this trait (Figure 2), underlining that chilling tolerance is a quantitative (polygenic) trait. However, genomic "hotspots" for chilling tolerance seem to exist, as indicated by several co-localizations of QTLs for chilling tolerance of photosynthesis among mapping populations. This suggests that for certain genomic regions, which are involved in the chilling tolerance of photosynthesis, a large variation exists across the maize germplasm. In particular, chromosome 2 harbors QTLs for chilling tolerance of photosynthesis in the ETH-DL3 × ETH-DH7 (Chapters 4-6), the ETH-EH3 × ETH-EL1 (J. Leipner, unpublished results) and the Ac7643 × Ac7729 mapping population (Fracheboud et al., 2002). Co-localizations between mapping populations were also found at chromosomes 1, 5 and 6. However, it still remains open whether these are real pleiotropic effects or whether a kind of cluster of genes important for chilling tolerance are present in these regions.

Complementation analysis of chilling-sensitive inbred lines, which were developed by divergent selection (Fracheboud et al., 1999), revealed that different causes of chilling sensitivity seem to exist among dent (D), flint (F) and part-exotic material (E) as well as within each of these heterotic groups (Box 2). When grown at suboptimal temperature, most of the hybrids of these lines showed a higher PSII operating efficiency ($F_{o'}/F_{m'}$) than the best parent. This indicates that the cause of chilling sensitivity was different between the parental lines and that chilling tolerance appears to be a dominant trait as it was also found for the major QTLs for chilling tolerance of photosynthesis (Chapter 4).

In order to disclose the whole genetic variation for chilling tolerance within the maize germplasm, additional mapping populations must be investigated or a large population suitable for association study must be developed and analyzed. Such a kind of collection was developed by us for wheat in order to study the

![Figure 2: Positions of major QTLs for chilling tolerance of photosynthesis, photosynthetic activity at optimal temperature, invertase activity, anthocyanin content, dry weight of field grown seedlings, plant height at flowering, drought tolerance at flowering as determined by leaf rolling and ear dry weight in the following mapping populations: ETH-DL3 × ETH-DH7 (orange; Chapters 4-6), IBM320 (green; Chapter 8), Lo964 × Lo1016 (blue; Hund et al., 2004), Ac7643 × Ac7729 (gray; Fracheboud et al., 2002), ETH-EH3 × ETH-EL1 (pink; J. Leipner, unpublished results) and ETH-DL7 × ETH-FH6 (yellow; S.K. Biradar, unpublished results). The positions of the QTLs are shown on the IBM2 2008 Neighbors consensus map; the beginning of each bin is indicated.](image-url)
Genetic causes of its winter hardiness by association mapping (Plassé, 2007). Based on a phylogenetic analysis of Swiss maize landraces, which show a large variation in their early vigor (Peter et al., 2009), a maize collection is being developed which will allow association studies in the future. The results for most of the generated hybrids showed higher \( F_q/F_m \) values then the best parent of this cross, especially when parental lines were from different heterotic groups, indicating a large genetic variation in chilling sensitivity of photosynthesis (Table 1). Furthermore, few maternal effects were detected.

Maternal effects
Quantitative genetic approaches usually do not consider maternal effects, but there are indications that the plastome may play a role in chilling tolerance. For example, it was found that proteins of the photosynthetic apparatus, which are encoded by the plastome, are reduced in their amount by growth at suboptimal temperature. Under these conditions however, nuclear encoded proteins were largely unaffected by the chilling stress (Nie and Baker, 1991). Based on the photosynthetic activity of reciprocal hybrids grown at suboptimal temperature (Chapter 3), maternal effects may be present in some of the crosses, even when the lines were derived from the same breeding pool. This was confirmed by a further analysis of another set of reciprocal hybrids which were derived from chilling-sensitive inbred lines (Box 2). The incidental appearance of maternal effects is also reflected in the fact that in some studies maternal effects were found (e.g., Eagles and Hardacre, 1979; Maryam and Jones, 1983), while in others this was not the case (Aidun et al., 1991). One reason for this observation might be the lower genetic variation that is known to be present in the chloroplast DNA (Bachmann, 1994). While this could explain maternal effects on the chilling tolerance of photosynthesis, maternal effects on biomass accumulation during heterotrophic growth at low temperature may be caused by the amount of seed reserves available for seedling growth. However, QTL analyses in the F3 generation of the ETH-DL3 × ETH-DH7 population revealed QTLs for single kernel weight which were not associated with QTLs for seedling shoot biomass (data not shown). This gives evidence that the seed weight does not necessarily affect the early vigor; an association between seed weight and early vigor was found only in some studies (Revilla et al., 1999) but not in others (Peter et al., 2009a).

Chilling tolerance of photosynthesis – Implications for crop growth
Co-localization of QTLs for photosynthesis-related traits and QTLs for biomass in seedlings grown at suboptimal temperature, which were found in the ETH- (Fracheboud et al., 2004) and the Lo-population (Hund et al., 2004), indicate that biomass accumulation at low temperature can be source limited. However, this seems to be true only at low or moderate light intensity, because under field conditions this co-localization was not present (Jompuk et al., 2005). Here, the amount of absorbed excitation seems to be sufficient to support growth even when leaves are photoinhibited, and other factors might become limiting for biomass accumulation. From the source side, the leaf area and the specific leaf area (SLA) are two factors that can influence the biomass accumulation. It was found that the QTLs for leaf area in seedlings grown under optimal temperature conditions in growth chambers (Jompuk, 2004) and

### Table 1: Complementation analysis based on the PSII operating efficiency \( F_q/F_m \) among chilling-sensitive (L) inbred lines derived from a dent (D), part-exotic (E) and flint (F) breeding pool (J. Leipner, unpublished results).

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<tr>
<th></th>
<th>DL3</th>
<th>DL4</th>
<th>EL1</th>
<th>EL2</th>
<th>FL5</th>
<th>FL8</th>
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<tr>
<td>♂</td>
<td>0.10</td>
<td>0.06</td>
<td>0.15</td>
<td>0.09</td>
<td>0.07</td>
<td>0.09</td>
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<td>n.d.</td>
<td>0.14</td>
<td>0.13</td>
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<td>n.d.</td>
<td>0.16</td>
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<tr>
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<td>0.11</td>
<td>0.02</td>
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<td>0.05</td>
</tr>
<tr>
<td>FL5</td>
<td>n.d.</td>
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<td>0.13</td>
<td>n.d.</td>
<td>0.10</td>
<td>0.07</td>
</tr>
<tr>
<td>FL8</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.09</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Note – Yellow background, higher \( F_q/F_m \) than mid-parent; orange background, higher \( F_q/F_m \) than best parent; bold, significant difference between the reciprocal crosses; n.d., not determined.
in the field (Jompuk et al., 2005) as well as the QTLs for SLA in plants grown at suboptimal temperature (Hund et al., 2005) show opposite additive effects compared to QTLs for photosynthesis-related traits. This indicates that more “sturdy plants” are characterized by a better photosynthetic performance. Such a phenotype, which is typical for stress tolerant plants, might come with a high cost, namely a reduced growth potential. This might not be the case at the seedling state since the QTL analysis revealed that genomic regions associated with small or thicker leaves (leaves with low SLA) are rather associated with larger biomass accumulation or do not show an effect on growth. However, later growth stages might be negatively affected in respect to growth by a good chilling tolerance of the seedling. This was indicated by a QTL for straw dry weight on chromosome 2 that mapped at the same position as QTLs for chilling tolerance of photosynthesis, but with contrasting additive effect (Leipner et al., 2008). It is suggested that the benefit of acclimation for survival comes at a cost (Hoffmann, 1995). However, the over-energization of the photosynthetic machinery as well as the accumulation of sugars during chilling stress (Sowinski et al., 1999) does not imply a limitation of assimilates for growth due to a high requirement of assimilates for defense systems. Moreover, the major QTL for chilling tolerance of photosynthesis was not associated with a QTL for biomass at harvest time, indicating that high chilling tolerance does not have a negative effect on later growth stages. More recently, it was found that an interaction exists between the DREB transcription factors, whose cold-induced expression was found also in maize (Chapter 11; Nguyen et al., 2009), and the gibberellic acid (GA) metabolism (Shan et al., 2007), resulting in growth retardation. By supplying GA to DREB transformed plants, growth could be restored without losing cold tolerance (Hsieu et al., 2002). On the other side, a good photosynthetic performance under optimal temperature, as it is manifested in the QTL on chromosome 3 in the ETH mapping population, seems to have a beneficial effect on growth and yield. Further supporting evidence was found by the QTL analysis in the F3 generation of the ETH-population, which revealed a QTL for single kernel weight at the above mentioned chromosomal region (data not shown). Since the allele responsible for a higher seed weight was not associated with a better early vigor (seedling biomass accumulation), it can be assumed that the higher source activity resulted in the increased kernel weight. Since the amount of seed reserves seems to be irrelevant at this growth stage, other factors might be limiting for seedling growth at low temperature, such as the availability of nitrogen or alterations in the phytohormone status of the seedling. Evidence for both was found in literature. Feil et al. (1993) observed a correlation between the activity of nitrate reductase, nitrate uptake and growth of maize. Interestingly, the position of the major QTL for seedling growth under field conditions is in the vicinity of the gene \textit{nnr2}, which codes for the nitrate reductase (Chapter 5). This suggests that the provision of ammonia might be the bottleneck of growth under field conditions. On the other hand, some phytohormones seem to have the potential to restore chilling-induced growth retardation. Such an effect was found for brassinolides (He et al., 1991).

Outlook

There is a particular interest in chilling tolerance due to changes in farming practices, such as earlier or no-till sowing, both of which expose seedlings to colder soils. The current level of adaptation of maize to cold climates is generally based on avoidance mechanisms, e.g. shortening of the plant vegetation to ensure a safe harvest in autumn. Nevertheless, these plants may be negatively affected by unpredictable cold spells at the beginning of the season. Although current climate models forecast that temperatures will increase, in some regions climate changes may be accompanied by a stronger fluctuation of the temperature and by a considerable decrease in precipitation during the growing season. A possible strategy to overcome this inadequate water supply is to avoid the drought period in summer by sowing plants much earlier. Thereby, these plants will be exposed to low temperature early in the season. Therefore, a high chilling tolerance will be essential.

The maize for the future needs to have the potential to develop a functional photosynthetic apparatus at low temperature in order to safely survive periods of low temperature, guaranteeing healthy plants. Furthermore, seedlings must be capable of quickly transiting to a state of high photosynthetic activity when conditions become more optimal. An efficient transformation of assimilates into biomass will require optimizing other processes that might be limiting, e.g. nitrogen uptake.

In particular, the identification of the key factors for the development of the photosynthetic apparatus under low temperature conditions will require optimized plant material and state of the art research methods. The generation of particular near isogenic lines (NILs) and the testing of this material with microarrays might be one possibility to achieve this goal. Thereby, the focus on developmental processes will be of high importance.
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**Curriculum vitae**

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